P1 1106519

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

December 18, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: ***60/418,030***
FILING DATE: ***October 11, 2002***
RELATED PCT APPLICATION NUMBER: *PCT/US03/32318*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

M. Sias

M. SIAS
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

10-15-02 04128 10_101102

A/PROV

PROVISIONAL APPLICATION COVER SHEET

Attorney Docket No.: CYTOP112P

First Named Inventor:

Commissioner for Patents
Box Provisional Patent Application
Washington, DC 20231

Sir: This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

INVENTOR(S)/APPLICANT(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)

TITLE OF INVENTION (280 characters max)

METHODS AND COMPOSITIONS UTILIZING 1,2,4-TRIAZIN-5-ONES

CORRESPONDENCE ADDRESS

Customer Number 022434

22434
PATENT AND TRADEMARK OFFICE

ENCLOSED APPLICATION PARTS (check all that apply)

- ☒ Specification — Number of Pages 62 — ☐ Other (specify) _____
- ☐ Drawing(s) — Number of Sheets _____
- ☐ Assignment and Assignment Recordation Cover Sheet (recording fee of $40.00 enclosed)

- ☐ Applicant is entitled to Small Entity Status under 37 C.F.R. § 1.27.
- ☐ A check or money order is enclosed to cover the Provisional filing fees. Provisional Filing Fee Amount ($) 160
- ☒ The commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to Deposit Account No. 501339 (Order No. CYTOP112P).

Respectfully Submitted,

SIGNATURE Lauren L. Stevens DATE October 11, 2002

TYPED NAME Lauren L. Stevens, Ph.D. REGISTRATION NO. 36,691

PROVISIONAL APPLICATION FILING ONLY

METHODS AND COMPOSITIONS UTILIZING 1,2,4-TRIAZIN-5-ONES

FIELD OF THE INVENTION

[0001] This invention relates to compounds which are inhibitors of the mitotic kinesin KSP and are useful in the treatment of cellular proliferative diseases, for example cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders, fungal disorders, and inflammation.

BACKGROUND OF THE INVENTION

[0002] Among the therapeutic agents used to treat cancer are the taxanes and vinca alkaloids, which act on microtubules. Microtubules are the primary structural element of the mitotic spindle. The mitotic spindle is responsible for distribution of replicate copies of the genome to each of the two daughter cells that result from cell division. It is presumed that disruption of the mitotic spindle by these drugs results in inhibition of cancer cell division, and induction of cancer cell death. However, microtubules form other types of cellular structures, including tracks for intracellular transport in nerve processes. Because these agents do not specifically target mitotic spindles, they have side effects that limit their usefulness.

[0003] Improvements in the specificity of agents used to treat cancer is of considerable interest because of the therapeutic benefits which would be realized if the side effects associated with the administration of these agents could be reduced. Traditionally, dramatic improvements in the treatment of cancer are associated with identification of therapeutic agents acting through novel mechanisms. Examples of this include not only the taxanes, but also the camptothecin class of topoisomerase I inhibitors. From both of these perspectives, mitotic kinesins are attractive targets for new anti-cancer agents.

[0004] Mitotic kinesins are enzymes essential for assembly and function of the mitotic spindle, but are not generally part of other microtubule structures, such as in nerve processes. Mitotic kinesins play essential roles during all phases of mitosis. These

enzymes are "molecular motors" that transform energy released by hydrolysis of ATP into mechanical force which drives the directional movement of cellular cargoes along microtubules. The catalytic domain sufficient for this task is a compact structure of approximately 340 amino acids. During mitosis, kinesins organize microtubules into the bipolar structure that is the mitotic spindle. Kinesins mediate movement of chromosomes along spindle microtubules, as well as structural changes in the mitotic spindle associated with specific phases of mitosis. Experimental perturbation of mitotic kinesin function causes malformation or dysfunction of the mitotic spindle, frequently resulting in cell cycle arrest and cell death.

[0005] Among the mitotic kinesins which have been identified is KSP. KSP belongs to an evolutionarily conserved kinesin subfamily of plus end-directed microtubule motors that assemble into bipolar homotetramers consisting of antiparallel homodimers. During mitosis KSP associates with microtubules of the mitotic spindle. Microinjection of antibodies directed against KSP into human cells prevents spindle pole separation during prometaphase, giving rise to monopolar spindles and causing mitotic arrest and induction of programmed cell death. KSP and related kinesins in other, non-human, organisms, bundle antiparallel microtubules and slide them relative to one another, thus forcing the two spindle poles apart. KSP may also mediate in anaphase B spindle elongation and focussing of microtubules at the spindle pole.

[0006] Human KSP (also termed HsEg5) has been described (Blangy, et al., Cell, 83:1159-69 (1995); Whitehead, et al., Arthritis Rheum., 39:1635-42 (1996); Galgio et al., J. Cell Biol., 135:339-414 (1996); Blangy, et al., J Biol. Chem., 272:19418-24 (1997); Blangy, et al., Cell Motil Cytoskeleton, 40:174-82 (1998); Whitehead and Rattner, J. Cell Sci., 111:2551-61 (1998); Kaiser, et al., JBC 274:18925-31 (1999); GenBank accession numbers: X85137, NM004523 and U37426), and a fragment of the KSP gene (TRIP5) has been described (Lee, et al., Mol Endocrinol., 9:243-54 (1995); GenBank accession number L40372). Xenopus KSP homologs (Eg5), as well as Drosophila KLP61 F/KRP1 30 have been reported.

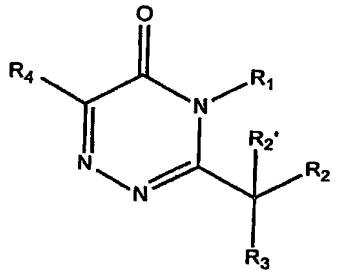
[0007] Mitotic kinesins are attractive targets for the discovery and development of novel antimitotic chemotherapeutics. Accordingly, it is an object of the present invention

to provide methods and compositions useful in the inhibition of KSP, a mitotic kinesin.

SUMMARY OF THE INVENTION

[0008] In accordance with the objects outlined above, the present invention provides compositions and methods that can be used to treat diseases of proliferating cells. The compositions are KSP inhibitors, particularly human KSP inhibitors.

[0009] In one aspect, the invention relates to methods for treating cellular proliferative diseases, for treating disorders by modulating the activity of KSP, and for inhibiting KSP kinesin. The methods employ compounds represented by Formula I:



Formula I

wherein:

R_1 is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, and optionally substituted heteroaralkyl;

R_2 and R_2' are independently chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, and optionally substituted heteroaralkyl; or R_2 and R_2' taken together form a 3- to 7-membered ring;

R_3 is selected from the group consisting of optionally substituted imidazolyl, optionally substituted imidazolinyl, $-NHR_5$; $-N(R_5)(COR_6)$; $-N(R_5)(SO_2R_{6a})$; and $-N(R_5)(CH_2R_{6b})$;

R_5 is chosen from hydrogen, optionally substituted alkyl, optionally substituted

aryl, optionally substituted aralkyl, optionally substituted heteroaryl, optionally substituted heteroaralkyl, and R_{11}-alkylene-;

R_6 is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, optionally substituted heteroaralkyl, R_7O- and R_7-NH-;

R_{6a} is chosen from optionally substituted alkyl, optionally substituted aryl, optionally substituted alkylaryl, optionally substituted heteroaryl, optionally substituted alkylheteroaryl, and R_7-NH-;

R_{6b} is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted alkylaryl, optionally substituted heteroaryl, and optionally substituted alkylheteroaryl;

R_7 is chosen from optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, and optionally substituted heteroaralkyl; and

R_{11} is chosen from optionally substituted alkoxy, amino, alkylamino, carboxy, dialkylamino, hydroxy, and optionally substituted N-heterocyclyl,

R_4 is independently chosen from hydrogen, optionally substituted alkyl, optionally substituted alkoxy, halogen, hydroxyl, nitro, cyano, dialkylamino, alkylsulfonyl, alkylsulfonamido, alkylthio, carboxyalkyl, carboxamido, aminocarbonyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaralkyl and optionally substituted heteroaryl;

including single stereoisomers, mixtures of stereoisomers, and the pharmaceutically acceptable salts thereof.

[0010] In one aspect, the invention relates to methods for treating cellular proliferative diseases and other disorders that can be treated by modulating KSP kinesin activity and for inhibiting KSP by the administration of a therapeutically effective amount of a compound of Formula I or a pharmaceutically acceptable salt thereof. Diseases and disorders that respond to therapy with compounds of the invention include cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders, fungal disorders and inflammation.

[0011] In another aspect, the invention relates to compounds useful in inhibiting KSP kinesin. The compounds have the structures shown above. The invention also relates to a pharmaceutical composition containing a therapeutically effective amount of a compound of Formula I or a pharmaceutically acceptable salt thereof admixed with at least one pharmaceutically acceptable excipient.

[0012] In an additional aspect, the present invention provides methods of screening for compounds that will bind to a KSP kinesin, for example compounds that will displace or compete with the binding of the compositions of the invention. The methods comprise combining a labeled compound of the invention, a KSP kinesin, and at least one candidate agent and determining the binding of the candidate bioactive agent to the KSP kinesin.

[0013] In a further aspect, the invention provides methods of screening for modulators of KSP kinesin activity. The methods comprise combining a composition of the invention, a KSP kinesin, and at least one candidate agent and determining the effect of the candidate bioactive agent on the KSP kinesin activity.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0014] As used in the present specification, the following words and phrases are generally intended to have the meanings as set forth below, except to the extent that the context in which they are used indicates otherwise. The following abbreviations and terms have the indicated meanings throughout:

Ac	=	acetyl
BNB	=	4-bromomethyl-3-nitrobenzoic acid
Boc	=	t-butyloxy carbonyl
Bu	=	butyl
c-	=	cyclo
CBZ	=	carbobenzoxy = benzyloxycarbonyl
DBU	=	diazabicyclo[5.4.0]undec-7-ene
DCM	=	dichloromethane = methylene chloride = CH_2Cl_2

DCE = dichloroethane
DEAD = diethyl azodicarboxylate
DIC = diisopropylcarbodiimide
DIEA = N,N-diisopropylethylamine
DMAP = 4-N,N-dimethylaminopyridine
DMF = N,N-dimethylformamide
DMSO = dimethyl sulfoxide
DVB = 1,4-divinylbenzene
EEDQ = 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
Et = ethyl
Fmoc = 9-fluorenylmethoxycarbonyl
GC = gas chromatography
HATU = O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMDS = hexamethyldisilazane
HOAc = acetic acid
HOBt = hydroxybenzotriazole
Me = methyl
mesyl = methanesulfonyl
MTBE = methyl t-butyl ether
NMO = N-methylmorpholine oxide
PEG = polyethylene glycol
Ph = phenyl
PhOH = phenol
PfP = pentafluorophenol
PPTS = pyridinium p-toluenesulfonate
Py = pyridine
PyBroP = bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
rt = room temperature
sat'd = saturated

s-	=	secondary
t-	=	tertiary
TBDMS	=	t-butyldimethylsilyl
TES	=	triethylsilyl
TFA	=	trifluoroacetic acid
THF	=	tetrahydrofuran
TMOF	=	trimethyl orthoformate
TMS	=	trimethylsilyl
tosyl	=	p-toluenesulfonyl
Trt	=	triphenylmethyl

[0015] **Alkyl** is intended to include linear, branched, or cyclic hydrocarbon structures and combinations thereof. **Lower alkyl** refers to alkyl groups of from 1 to 5 carbon atoms. Examples of lower alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, s-and t-butyl and the like. Preferred alkyl groups are those of C_{20} or below. More preferred alkyl groups are those of C_{13} or below. Cycloalkyl is a subset of alkyl and includes cyclic hydrocarbon groups of from 3 to 13 carbon atoms. Examples of cycloalkyl groups include c-propyl, c-butyl, c-pentyl, norbornyl, adamantyl and the like. In this application, alkyl refers to alkanyl, alkenyl and alkynyl residues; it is intended to include cyclohexylmethyl, vinyl, allyl, isoprenyl and the like. Alkylene is another subset of alkyl, referring to the same residues as alkyl, but having two points of attachment. Examples of alkylene include ethylene ($-CH_2CH_2-$), propylene ($-CH_2CH_2CH_2-$), dimethylpropylene ($-CH_2C(CH_3)_2CH_2-$) and cyclohexylpropylene ($-CH_2CH_2CH(C_6H_{13})-$). When an alkyl residue having a specific number of carbons is named, all geometric isomers having that number of carbons are intended to be encompassed; thus, for example, "butyl" is meant to include n-butyl, sec-butyl, isobutyl and t-butyl; "propyl" includes n-propyl and isopropyl.

[0016] **Alkoxy** or **alkoxyl** refers to the group -O-alkyl, preferably including from 1 to 8 carbon atoms of a straight, branched, cyclic configuration and combinations thereof

attached to the parent structure through an oxygen. Examples include methoxy, ethoxy, propoxy, isopropoxy, cyclopropyloxy, cyclohexyloxy and the like. Lower-alkoxy refers to groups containing one to four carbons.

[0017] **Acyl** refers to groups of from 1 to 8 carbon atoms of a straight, branched, cyclic configuration, saturated, unsaturated and aromatic and combinations thereof, attached to the parent structure through a carbonyl functionality. One or more carbons in the acyl residue may be replaced by nitrogen, oxygen or sulfur as long as the point of attachment to the parent remains at the carbonyl. Examples include acetyl, benzoyl, propionyl, isobutyryl, t-butoxycarbonyl, benzyloxycarbonyl and the like. Lower-acyl refers to groups containing one to four carbons.

[0018] **Amino** refers to the group $-NH_2$. **Substituted amino** refers to the group -NHR or -NRR where each R is independently selected from the group: optionally substituted alkyl, optionally substituted alkoxy, optionally substituted amino, optionally substituted aryl, optionally substituted heteroaryl, optionally substituted heterocyclyl, aminocarbonyl, acyl, alkoxycarbonyl, sulfanyl, sulfinyl and sulfonyl, e.g., diethylamino, methylsulfonylamino, furanyl-oxy-sulfonamino.

[0019] **Aryl** and **heteroaryl** mean a 5- or 6-membered aromatic or heteroaromatic ring containing 0-4 heteroatoms selected from O, N, or S; a bicyclic 9- or 10-membered aromatic or heteroaromatic ring system containing 0-4 (or more) heteroatoms selected from O, N, or S; or a tricyclic 12- to 14-membered aromatic or heteroaromatic ring system containing 0-4 (or more) heteroatoms selected from O, N, or S. The aromatic 6- to 14-membered carbocyclic rings include, e.g., benzene, naphthalene, indane, tetralin, and fluorene and the 5- to 10-membered aromatic heterocyclic rings include, e.g., imidazole, pyridine, indole, thiophene, benzopyranone, thiazole, furan, benzimidazole, quinoline, isoquinoline, quinoxaline, pyrimidine, pyrazine, tetrazole and pyrazole.

[0020] **Aralkyl** refers to a residue in which an aryl moiety is attached to the parent structure via an alkyl residue. Examples include benzyl, phenethyl, phenylvinyl, phenylallyl and the like. **Heteroaralkyl** refers to a residue in which a heteroaryl moiety is attached to the parent structure via an alkyl residue. Examples include furanylmethyl, pyridinylmethyl, pyrimidinylethyl and the like.

[0021] **Aralkoxy** refers to the group -O-aralkyl. Similarly, **heteroaralkoxy** refers to the group –O-heteroaralkyl; **aryloxy** refers to the group –O-aryl; and **acyloxy** refers to the group –O-acyl.

[0022] **Halogen** or **halo** refers to fluorine, chlorine, bromine or iodine. Fluorine, chlorine and bromine are preferred. Dihaloaryl, dihaloalkyl, trihaloaryl etc. refer to aryl and alkyl substituted with a plurality of halogens, but not necessarily a plurality of the same halogen; thus 4-chloro-3-fluorophenyl is within the scope of dihaloaryl.

[0023] **Heterocycle** means a cycloalkyl or aryl residue in which one to four of the carbons is replaced by a heteroatom such as oxygen, nitrogen or sulfur. Examples of heterocycles that fall within the scope of the invention include imidazoline, pyrrolidine, pyrazole, pyrrole, indole, quinoline, isoquinoline, tetrahydroisoquinoline, benzofuran, benzodioxan, benzodioxole (commonly referred to as methylenedioxyphenyl, when occurring as a substituent), tetrazole, morpholine, thiazole, pyridine, pyridazine, piperidine, pyrimidine, thiophene, furan, oxazole, oxazoline, isoxazole, dioxane, tetrahydrofuran and the like. "N-heterocyclyl" refers to a nitrogen-containing heterocycle as a substituent residue. The term heterocyclyl encompasses heteroaryl, which is a subset of heterocyclyl. Examples of N-heterocyclyl residues include 4-morpholinyl, 4-thiomorpholinyl, 1-piperidinyl, 1-pyrrolidinyl, 3-thiazolidinyl, piperazinyl and 4-(3,4-dihydrobenzoxazinyl). Examples of substituted heterocyclyl include 4-methyl-1-piperazinyl and 4-benzyl-1-piperidinyl.

[0024] **Optional** or **optionally** means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstances occurs and instances in which it does not. For example, "optionally substituted alkyl" means either "alkyl" or "substituted alkyl" as defined herein. It will be understood by those skilled in the art with respect to any group containing one or more substituents that such groups are not intended to introduce any substitution or substitution patterns (e.g., substituted alkyl includes optionally substituted cycloalkyl groups, which in turn are defined as including optionally substituted alkyl groups, potentially *ad infinitum*) that are sterically impractical and/or synthetically non-feasible and/or inherently unstable.

[0025] **Substituted alkoxy** refers to the group -O-(substituted alkyl). One preferred substituted alkoxy group is "polyalkoxy" or -O-(optionally substituted alkylene)-(optionally substituted alkoxy), and includes groups such as $-OCH_2CH_2OCH_3$, and glycol ethers such as polyethyleneglycol and $-O(CH_2CH_2O)_xCH_3$, where x is an integer of about 2-20, preferably about 2-10, and more preferably about 2-5. Another preferred substituted alkoxy group is hydroxyalkoxy or $-OCH_2(CH_2)_yOH$, where y is an integer of about 1-10, preferably about 1-4.

[0026] **Substituted**- alkyl, aryl, heteroaryl and heterocyclyl refer respectively to alkyl, aryl, heteroaryl and heterocyclyl wherein one or more (up to about 5, preferably up to about 3) hydrogen atoms are replaced by a substituent independently selected from the group: optionally substituted alkyl (e.g., fluoroalkyl), optionally substituted alkoxy, alkylenedioxy (e.g. methylenedioxy), optionally substituted amino (e.g., alkylamino and dialkylamino),optionally substituted amidino, optionally substituted aryl (e.g., phenyl), optionally substituted aralkyl (e.g., benzyl), optionally substituted aryloxy (e.g., phenoxy), optionally substituted aralkoxy (e.g., benzyloxy), carboxy (-COOH), carboalkoxy (i.e., acyloxy or -OOCR), carboxyalkyl (i.e., esters or -COOR), carboxamido, aminocarbonyl, benzyloxycarbonylamino (CBZ-amino), cyano, acyl, halogen, hydroxy, optionally substituted heteroaryl, optionally substituted heteroaralkyl, optionally substituted heteroaryloxy, optionally substituted heteroaralkoxy, nitro, sulfanyl, sulfinyl, sulfonyl, and thio.

[0027] **Sulfanyl** refers to the groups: -S-(optionally substituted alkyl), -S-(optionally substituted aryl), -S-(optionally substituted heteroaryl), and -S-(optionally substituted heterocyclyl).

[0028] **Sulfinyl** refers to the groups: -S(O)-H, -S(O)-(optionally substituted alkyl), -S(O)-optionally substituted aryl), -S(O)-(optionally substituted heteroaryl), -S(O)-(optionally substituted heterocyclyl); and -S(O)-(optionally substituted amino).

[0029] **Sulfonyl** refers to the groups: $-S(O_2)$-H, $-S(O_2)$-(optionally substituted alkyl), $-S(O_2)$-optionally substituted aryl), $-S(O_2)$-(optionally substituted heteroaryl), $-S(O_2)$-(optionally substituted heterocyclyl) ,$-S(O_2)$-(optionally substituted alkoxy), $-S(O_2)$-optionally substituted aryloxy), $-S(O_2)$-(optionally substituted heteroaryloxy),

-S(O_2)-(optionally substituted heterocyclyloxy); and -S(O_2)-(optionally substituted amino).

[0030] **Pharmaceutically acceptable acid addition salt** refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

[0031] **Pharmaceutically acceptable base addition salts** include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.

[0032] Many of the compounds described herein contain one or more asymmetric centers (e.g. the carbon to which R_2 and R_2' are attached) and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)-. The present invention is meant to include all such possible isomers, including racemic mixtures, optically pure forms and intermediate mixtures. Optically active (R)- and (S)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques. When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless specified otherwise, it is intended that the compounds include both E and Z geometric isomers. Likewise, all tautomeric forms and rotational isomers are also intended to be included.

[0033] When desired, the R- and S-isomers may be resolved by methods known

to those skilled in the art, for example by formation of diastereoisomeric salts or complexes which may be separated, for example, by crystallisation; via formation of diastereoisomeric derivatives which may be separated, for example, by crystallisation, gas-liquid or liquid chromatography; selective reaction of one enantiomer with an enantiomer-specific reagent, for example enzymatic oxidation or reduction, followed by separation of the modified and unmodified enantiomers; or gas-liquid or liquid chromatography in a chiral environment, for example on a chiral support, such as silica with a bound chiral ligand or in the presence of a chiral solvent. It will be appreciated that where the desired enantiomer is converted into another chemical entity by one of the separation procedures described above, a further step may be required to liberate the desired enantiomeric form. Alternatively, specific enantiomer may be synthesized by asymmetric synthesis using optically active reagents, substrates, catalysts or solvents, or by converting one enantiomer to the other by asymmetric transformation.

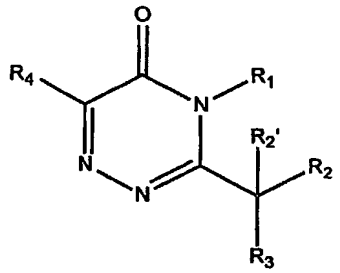
Compounds of the Present Invention

[0034] The present invention is directed to a class of novel compounds, based on a core 1,2,4-triazin-5-one structure, that are modulators of mitotic kinesins. By inhibiting or modulating mitotic kinesins, but not other kinesins (e.g., transport kinesins), specific inhibition of cellular proliferation is accomplished. While not intending to be bound by any theory, the present invention capitalizes on the finding that perturbation of mitotic kinesin function causes malformation or dysfunction of mitotic spindles, frequently resulting in cell cycle arrest and cell death. The methods of inhibiting a human KSP kinesin comprise contacting an inhibitor of the invention with a KSP kinesin, particularly human KSP kinesins, including fragments and variants of KSP. The inhibition can be of the ATP hydrolysis activity of the KSP kinesin and/or the mitotic spindle formation activity, such that the mitotic spindles are disrupted. Meiotic spindles may also be disrupted.

[0035] An object of the present invention is to develop inhibitors and modulators of mitotic kinesins, in particular KSP, for the treatment of disorders associated with cell proliferation. Traditionally, dramatic improvements in the treatment of cancer, one type

of cell proliferative disorder, have been associated with identification of therapeutic agents acting through novel mechanisms. Examples of this include not only the taxane class of agents that appear to act on microtubule formation, but also the camptothecin class of topoisomerase I inhibitors. The compositions and methods described herein can differ in their selectivity and are preferably used to treat diseases of proliferating cells, including, but not limited to cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders, fungal disorders and inflammation.

[0036] Accordingly, the present invention relates to methods employing compounds represented by Formula I:



Formula I

wherein:

R_1 is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, and optionally substituted heteroaralkyl;

R_2 and R_2' are independently chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, and optionally substituted heteroaralkyl; or R_2 and R_2' taken together form a 3- to 7-membered ring;

R_3 is selected from the group consisting of optionally substituted imidazolyl, optionally substituted imidazolinyl, $-NHR_5$; $-N(R_5)(COR_6)$; $-N(R_5)(SO_2R_{6a})$; and $-N(R_5)(CH_2R_{6b})$;

R_5 is chosen from hydrogen, optionally substituted alkyl, optionally substituted

aryl, optionally substituted aralkyl, optionally substituted heteroaryl, optionally substituted heteroaralkyl, and R_{11}-alkylene-;

R_6 is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, optionally substituted heteroaralkyl, R_7O- and R_7-NH-;

R_{6a} is chosen from optionally substituted alkyl, optionally substituted aryl, optionally substituted alkylaryl, optionally substituted heteroaryl, optionally substituted alkylheteroaryl, and R_7-NH-;

R_{6b} is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted alkylaryl, optionally substituted heteroaryl, and optionally substituted alkylheteroaryl;

R_7 is chosen from optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, and optionally substituted heteroaralkyl; and

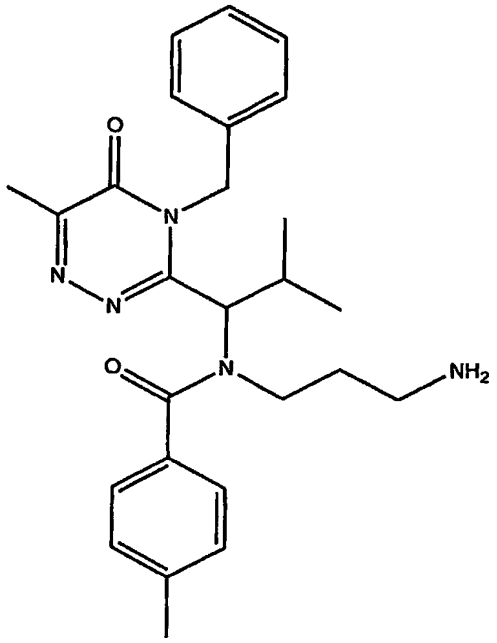
R_{11} is chosen from optionally substituted alkoxy, amino, alkylamino, carboxy, dialkylamino, hydroxy, and optionally substituted N-heterocyclyl,

R_4 is independently chosen from hydrogen, optionally substituted alkyl, optionally substituted alkoxy, halogen, hydroxyl, nitro, cyano, dialkylamino, alkylsulfonyl, alkylsulfonamido, alkylthio, carboxyalkyl, carboxamido, aminocarbonyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaralkyl and optionally substituted heteroaryl;

including single stereoisomers, mixtures of stereoisomers, and the pharmaceutically acceptable salts thereof. In a particularly preferred embodiment, the stereogenic center to which R_2 and $R_{2'}$ are attached is of the R configuration.

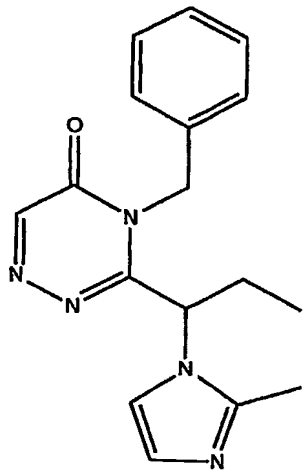
Nomenclature

[0037] The compounds of Formula I are named and numbered as described below. For example, the compound:



i.e., the compound according to Formula I where R_1 is benzyl, R_2 is propyl (or i-propyl), $R_{2'}$ is hydrogen; R_3 is $-N(R_5)(COR_6)$; R_4 is methyl; R_5 is aminopropyl; and R_6 is p-tolyl can be named N-(3-Amino-propyl)-N-[1-(4-benzyl-6-methyl-5-oxo-4,5-dihydro-[1,2,4]triazin-3-yl)-2-methyl-propyl]-4-methyl-benzamide.

[0038] Likewise, the compound



i.e., the compound according to Formula I where R_1 is benzyl, R_2 is ethyl, $R_{2'}$ is hydrogen; R_3 is substituted imidazolyl; and R_4 is hydrogen can be named 4-benzyl-3-[1-(2-methyl-imidazol-1-yl)-propyl]-4*H*-[1,2,4]triazin-5-one.

Synthetic Reaction Parameters

[0039] The terms "solvent", "inert organic solvent" or "inert solvent" mean a solvent inert under the conditions of the reaction being described in conjunction therewith [including, for example, benzene, toluene, acetonitrile, tetrahydrofuran ("THF"), dimethylformamide ("DMF"), chloroform, methylene chloride (or dichloromethane), diethyl ether, methanol, pyridine and the like]. Unless specified to the contrary, the solvents used in the reactions of the present invention are inert organic solvents.

[0040] The term "q.s." means adding a quantity sufficient to achieve a stated function, e.g., to bring a solution to the desired volume (i.e., 100%).

[0041] Isolation and purification of the compounds and intermediates described herein can be affected, if desired, by any suitable separation or purification procedure such as, for example, filtration, extraction, crystallization, column chromatography, thin-layer chromatography or thick-layer chromatography, or a combination of these procedures. Specific illustrations of suitable separation and isolation procedures can be had by reference to the examples hereinbelow. However, other equivalent separation or

isolation procedures can, of course, also be used.

Synthesis of the Compounds of Formula I

[0042] The compounds of Formula I can be prepared by following the procedures described with reference to the Reaction Schemes below.

Brief Description of Reaction Schemes

[0043] Reaction Scheme 1 illustrates synthesis of compounds of Formula I wherein R_3 is $-N(R_5)(COR_6)$.

[0044] Reaction Scheme 2 illustrates a synthesis of compounds of Formula I wherein R_3 is $-N(R_5)(SO_2R_6)$.

[0045] Reaction Scheme 3 illustrates a synthesis of compounds of Formula I wherein R_3 is $-N(R_5)(CH_2R_6)$.

[0046] Reaction Scheme 4 illustrates a synthesis of compounds of Formula I wherein R_3 is optionally substituted imidazolyl.

[0047] Reaction Scheme 5 illustrates an alternative synthesis of compounds of Formula I wherein R_3 is optionally substituted imidazolyl.

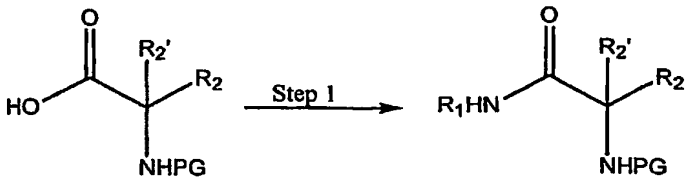
[0048] Reaction Scheme 6 illustrates a synthesis of compounds of Formula I wherein R_3 is optionally substituted imidazolinyl.

[0049] Reaction Scheme 7 illustrates an alternative synthesis of compounds of Formula I wherein R_3 is optionally substituted imidazolinyl.

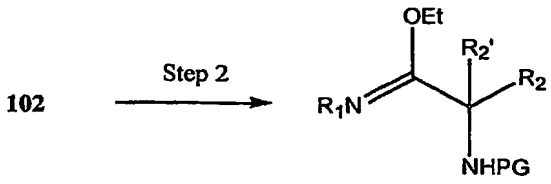
Starting Materials

[0050] The protected α-amino acids of Formula 101 are commercially available, e.g., from Aldrich Chemical Company, Milwaukee, WI. Other reactants are likewise commercially available or may be readily prepared by those skilled in the art using commonly employed synthetic methodology.

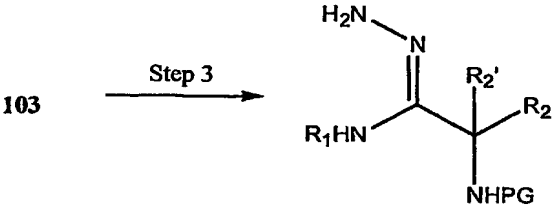
Reaction Scheme 1



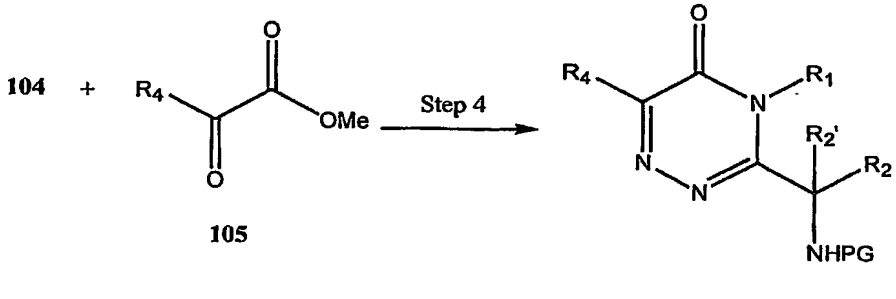
101 102



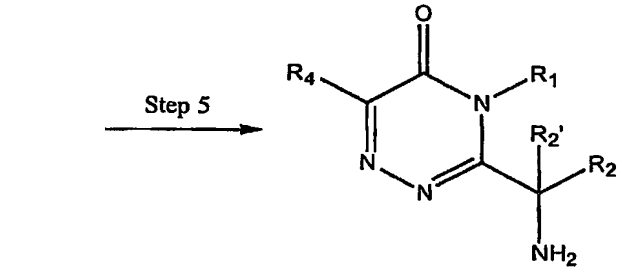
103



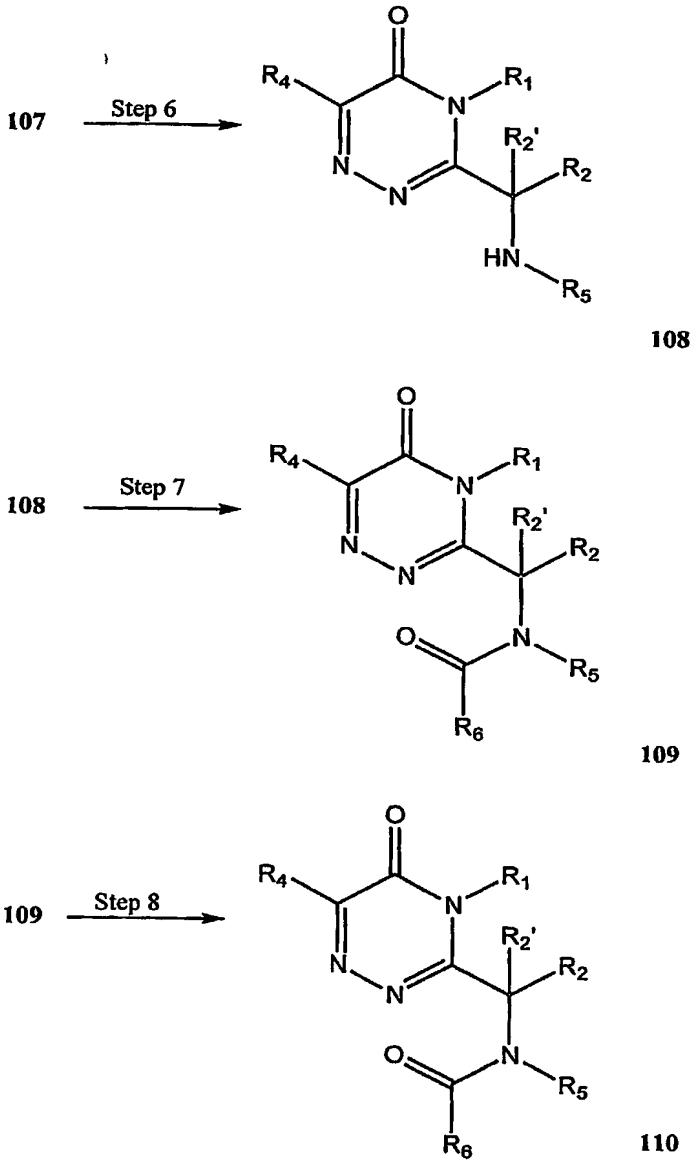
104



105 106



107



Preparation of Formula 102

[0051] Referring to Reaction Scheme 1, Step 1, to a solution of a suitably protected α-amino acid (the compound of Formula 101, preferably wherein protecting group, PG, is CBZ) in an inert organic solvent (such as THF) is added ethyl chloroformate and a tertiary amine base (such as triethylamine or DIEA) at 0 °C. The reaction micxture is stirred under nitrogen. After 1 hour, a primary amine with the

formula of R_1-NH_2 (where R_1 is described as above) is added over 5-10 minutes. Upon completion of addition, the reaction solution is allowed to warm to room temperature. The reaction mixture is stirred for 1-4 hours. The optionally substituted α-amino amide (the compound of Formula 102) is isolated and purified.

Preparation of Formula 103

[0052] Referring to Reaction Scheme 1, Step 2, a solution of a compound of Formula 102 and Meerwein's salt (such as triethyloxonium hexafluorophosphate) in dichloromethane is stirred for 14-24 hours. The product, an imidate of Formula 103, is isolated and used in the next step without further purifaction.

Preparation of Formula 104

[0053] Referring to Reaction Scheme 1, Step 3, to a solution of an imidate of Formula 103 in a polar, aprotic solvent (such as THF) is added a solution of hydrazine in THF. The reaction mixture is stirred for about 4-9 hours. The product, a hydrazidine of Formula 104, is isolated and purified.

Preparation of Formula 106

[0054] Referring to Reaction Scheme 1, Step 4, a solution of a hydrazidine of Formula 104 and a reagent of Formaula 105 in EtOH or toluene is refluxed for 4-10 hours. AcOH (catalytic amount) is added. Refluxing continues for additional 6-10 hours. The product, a 1,2,4-triazin-5-one of Formula 106, is isolated and purified.

Preparation of Formula 107

[0055] Referring to Reaction Scheme 1, Step 5, the amino-protecting group of a compound of Formula 106 is removed. For example, to a solution of a 1,2,4-triazin-5-one of Formula 106 wherein the amino protecting group, PG, is CBZ in a polar solvent such as methanol is added 10% Pd on carbon. The reaction mixture is stirred under a stream of H_2 (20-40 psi) for 40 minutes to 2 hours. The product, a compound of Formula 107, is isolated and used in the next step without further purification. One of skill in the

art will readily appreciate that the removal of other protecting groups can be accomplished using conditions known in the art. See, e.g., Greene, et al. *supra*.

Preparation of Formula 108

[0056] Referring to Reaction Scheme 1, Step 6, to a solution of a 1,2,4-triazin-5-one of Formula 107 is added successively a slight excess (preferably about 1.2 equivalents) of an aldehyde comprising R_5, (i.e., a compound having the formula R_5.CHO where $R_5.CH_2$- is equivalent to R_5 and R_5 is as described above or is a protected precursor to such a substituent, e.g., (3-oxo-propyl)-carbamic acid *tert*-butyl ester) and a reducing agent such as sodium triacetoxyborohydride. The resulting mixture is stirred for several hours. The product, a 1,2,4-triazin-5-one of Formula 108, is isolated and purified.

Preparation of Formula 109

[0057] Referring to Reaction Scheme 1, Step 7, to a solution of a 1,2,4-triazin-5-one of Formula 108 and an amine base such as diisopropylethylamine in a polar, aprotic solvent such as dichloromethane is added an R_6 acyl chloride (such as Cl-C(O)-R_6 where R_6 is as described above). The resulting solution is stirred under nitrogen at room temperature for several hours. The product, a 1,2,4-triazin-5-one of Formula 109, is isolated and purified.

Preparation of Formula 110

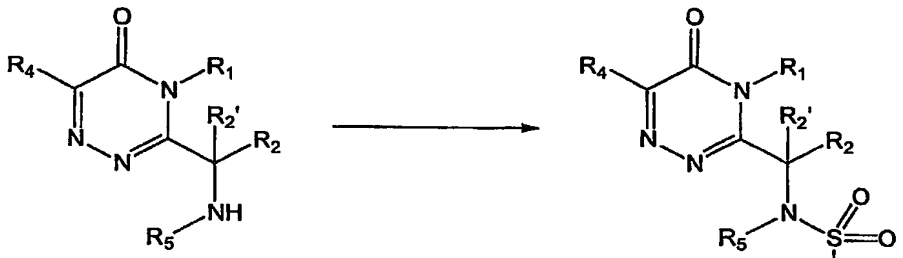
[0058] Optionally, any protecting groups on a compound of Formula 109 are then removed. For example, if R_5 comprises a protected amine wherein the protecting group is a Boc group, then referring to Reaction Scheme 1, Step 8, to a solution of a 1,2,4-triazin-5-one of Formula 110 in a polar, aprotic solvent such as dichloromethane is added trifluoroacetic acid, while maintaining the reaction at about room temperature. The reaction is monitored, e.g., by TLC. Upon completion, the product, a 1,2,4-triazin-5-one of Formula 110, is isolated and purified.

Preparation of Optically Active Compounds of Formula 107

[0059] In certain compounds of the invention, a particular stereo configuration (such as the (R) isomer) may be preferred at the stereogenic center to which R_2 is attached. The optically active compound can be prepared by methods known in the art. For example, an amine of Formula 107 is dissolved in an inert organic solvent (such as IPA) and warmed to 60°C. In a separate vessel, a resolving agent (such as dibenzoyl-D-tartaric acid) is dissolved, preferably in the same warm solvent, and then quickly added (with agitation) to the warm amine solution. The reaction mixture is left to crystallize by cooling to room temperature over 16 hours under continuing agitation. The desired isomer, e.g., the (R) isomer illustrated as Formula 107a, is isolated and purified.

[0060] For the sake of brevity in the remaining description of the synthesis of compounds of Formula I, it should be understood that either single isomer or a mixture of isomers may be employed to give the corresponding product.

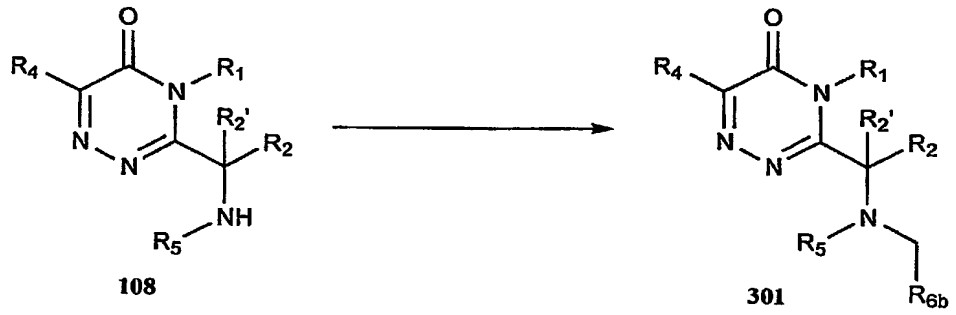
Reaction Scheme 2



[0061] Referring to Reaction Scheme 2, to a solution of a 1,2,4-triazin-5-one of Formula 108 and an amine base (such as diisopropylethylamine) in a polar, aprotic solvent (such as dichloromethane) is added a compound having the formula $Cl\text{-}S(O)_2\text{-}R_{6a}$ or $O\text{-}(S(O)_2\text{-}R_{6a})_2$ where R_{6a} is as described above. The resulting solution is stirred under nitrogen at room temperature for several hours. The product, a 1,2,4-triazin-5-one of

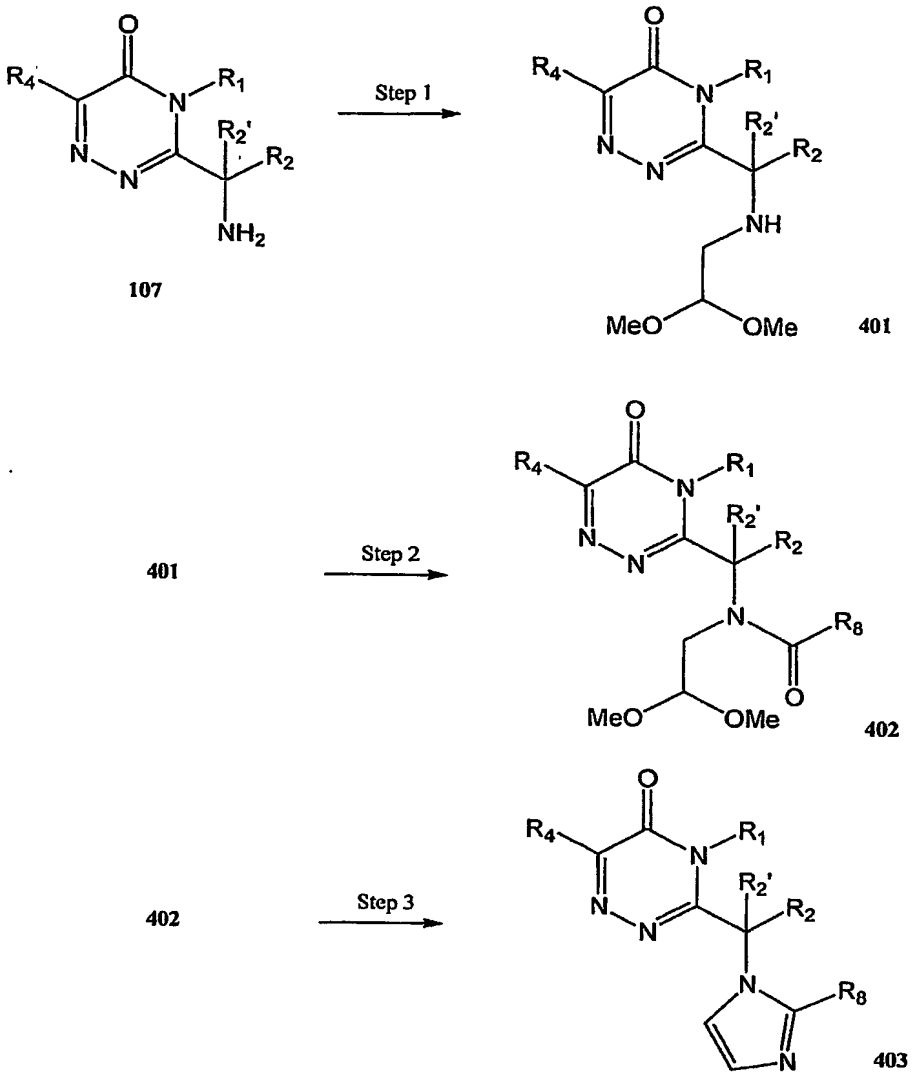
Formula 201 is isolated and purified.

Reaction Scheme 3



[0062] Referring to Reaction Scheme 3, to a solution of a 1,2,4-triazin-5-one of Formula 108 and an amine base (such as diisopropylethylamine) in a polar, aprotic solvent (such as dichloromethane) is added a compound having the formula $Br\text{-}CH_2\text{-}R_{6b}$ or $Cl\text{-}CH_2\text{-}R_{6b}$ where R_{6b} is as described above. The resulting solution is stirred under nitrogen at room temperature or with heat for several hours. The product, a 1,2,4-triazin-5-one of Formula 301 is isolated and purified.

Reaction Scheme 4



Preparation of Formula 401

[0063] Referring to Reaction Scheme 4, Step 1, to an optionally substituted compound of Formula 107 dissolved in a polar, aprotic solvent (such as DMF) in the presence of a base (such as potassium carbonate) is added one equivalent of an optionally substituted suitably protected aldehyde wherein such aldehyde further comprises a leaving group, preferably, a halide. The solution is heated at reflux, monitoring

completion of the reaction (e.g., by TLC). The reaction mixture is cooled and the corresponding, optionally substituted 1,2,4-triazin-5-one of Formula 401 is isolated and purified.

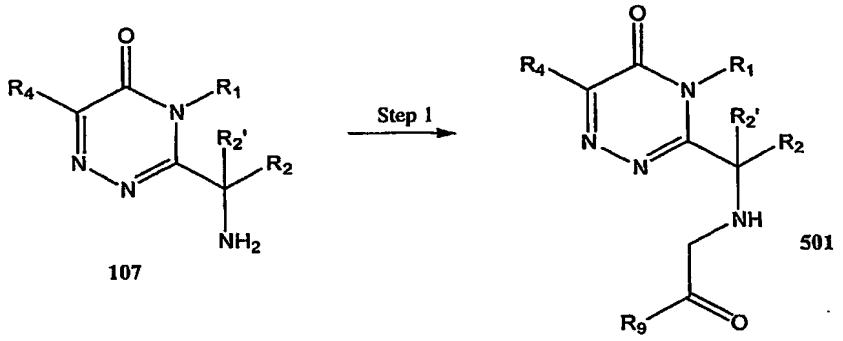
Preparation of Formula 402

[0064] Referring to Reaction Scheme 4, Step 2, to an optionally substituted compound of Formula 402 in an inert solvent (such as dichloromethane) in the presence of about 1.5 molar equivalents of an amine base (such as triethylamine) is added about 1.5 molar equivalents of an R_8 acid chloride, such as, Cl-C(O)-R_8, where R_8 is as described below. The reaction takes place, with stirring, at room temperature over a period of 4 to 24 hours. Completion is monitored, e.g., by TLC. The corresponding compound of Formula 402 is isolated and purified.

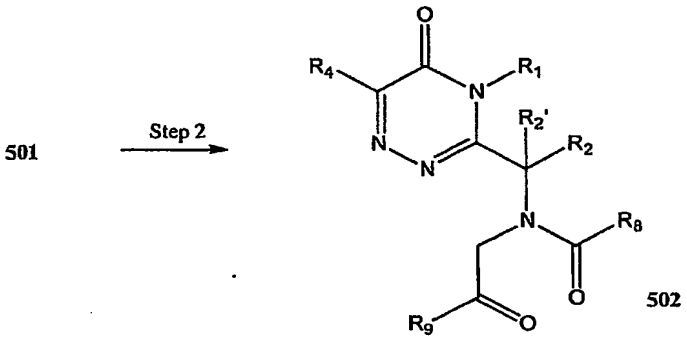
Preparation of Formula 403

[0065] Referring to Reaction Scheme 4, Step 3, a solution of a compound of Formula 402 and an excess of ammonium acetate in acetic acid is heated at reflux for 1-4 hours. Completion is monitored, e.g., by TLC. The corresponding compound of Formula 403 is isolated and purified.

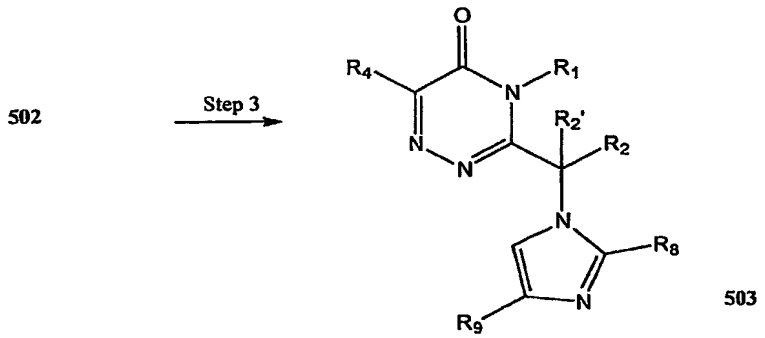
Reaction Scheme 5



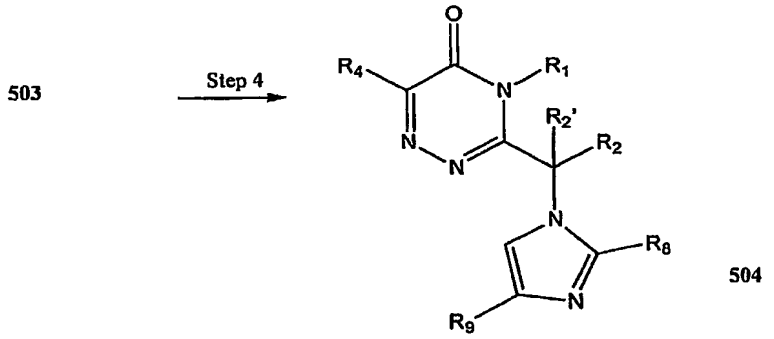
107 501



502



503



504

Preparation of Formula 501

[0066] Referring to Reaction Scheme 5, Step 1, a suspension of a compound of Formula 107, an alpha-haloketone reagent of the Formula $R_9(CO)CH_2X$ wherein X is a halide, and about an equivalent of a base, such as potassium carbonate in a polar, aprotic solvent such as DMF is stirred at room temperature. The reaction is diluted with water and the resulting solid, a compound of Formula 501, is used in the subsequent step without further purification.

Preparation of Formula 502

[0067] Referring to Reaction Scheme 5, Step 2, a solution of the compound of Formula 501, about an equivalent of an amine base, such as triethylamine and about an equivalent of an acid chloride (such as a compound of Formula R_8-COCl) in an organic solvent such as methylene chloride is stirred at room temperature for several hours. Completion is monitored, e.g., by TLC. The corresponding compound of Formula 502 is isolated and purified.

Preparation of Formula 503

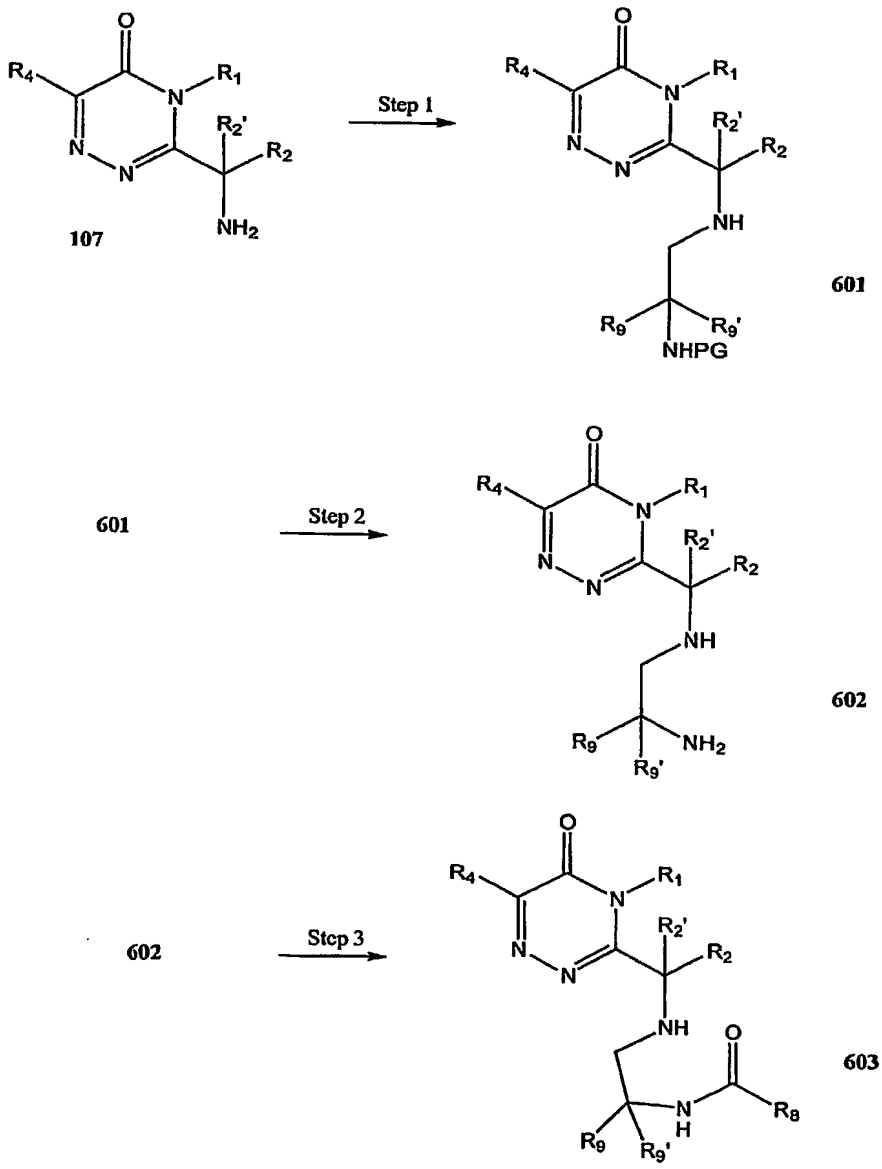
[0068] Referring to Reaction Scheme 5, Step 3, a solution of a compound of Formula 502 and an excess of ammonium acetate in acetic acid is heated at reflux using a Dean-Stark trap and condenser. Completion is monitored, e.g., by TLC. The corresponding compound of Formula 503 is isolated and purified.

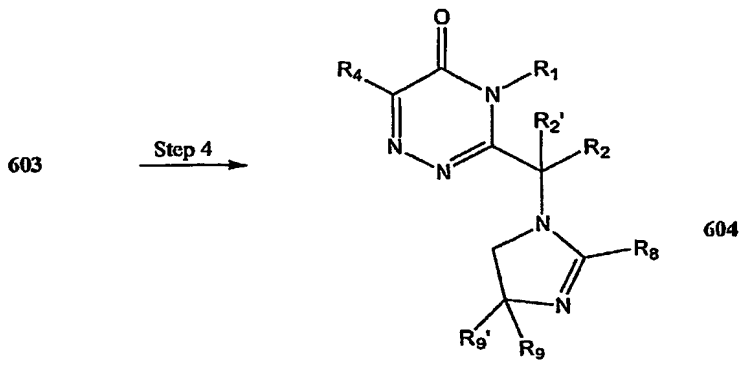
Preparation of Formula 504

[0069] Optionally, any protecting groups on compounds of Formula 507 are then removed. For example, if a phthalidmide protecting group is used, it may be removed as follows. A solution of a compound of Formula 507 and an excess of anhydrous hydrazine in a polar, protic solvent such as ethanol is heated at reflux. The reaction is cooled to about 5°C and any precipitate is filtered off. The filtrate is concentrated in vacuo and purified to yield a compound of Formula 509. One of skill in the art will

appreciate that other conditions may be used to remove other protecting groups.

Reaction Scheme 6





Preparation of Formula 601

[0070] Referring to Reaction Scheme 6, Step 1, reductive amination of amines of Formula 107 (prepared as described in WO 0130768) with an optionally substituted, aldehyde-containing carbamic acid ester (Seki *et. al. Chem. Pharm. Bull.* 1996, *44*, 2061) gives urethane intermediates. Removal of the Boc protecting group furnishes an amine of Formula 601.

[0071] More specifically, to a solution of a compound of Formula 107 and an equivalent of a suitably protected aldehyde (Seki *et. al. Chem. Pharm. Bull.* 1996, *44*, 2061) in dichloromethane is added a slight excess of a reducing agent, such as sodium triacetoxyborohydride. The resultant cloudy mixture is maintained at ambient temperature. Completion is monitored, e.g., by TLC. The corresponding compound of Formula 602 is isolated and used in the subsequent step without purification.

Preparation of Formula 602

[0072] Referring to Reaction Scheme 6, Step 2, to a solution of a compound of Formula 703 in a polar, aprotic solvent such as dichloromethane is added a strong acid such as trifluoroacetic acid. The resultant solution is maintained at ambient temperature overnight and concentrated under reduced pressure. The residue is isolated to give a compound of Formula 602 which is used in the subsequent step without purification.

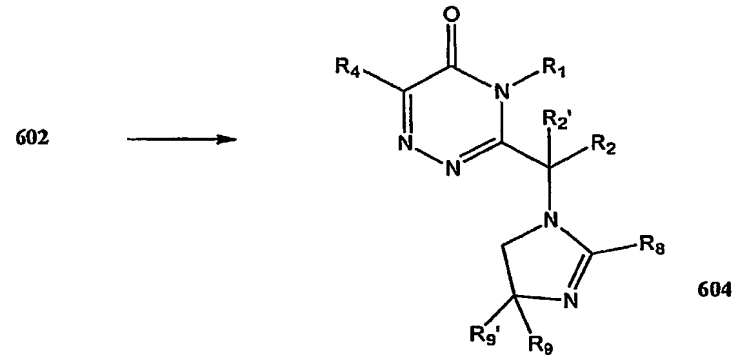
Preparation of Formula 603

[0073] Referring to Reaction Scheme 6, Step 3, to a solution of a compound of Formula 602 in a polar, aprotic solvent such as dichloromethane is added an excess, preferably about two equivalents of an amine base such as triethylamine, followed by about an equivalent or slight excess of an acid chloride. The resultant solution is stirred at ambient temperature for about 3 hours. Completion is monitored, e.g., by TLC. The corresponding compound of Formula 603 is isolated and purified.

Preparation of Formula 604

[0074] Referring to Reaction Scheme 6, Step 4, a solution of a compound of Formula 707 in an excess of phosphorus oxychloride is heated at reflux. After 8 hours, the reaction mixture is allowed to cool to ambient temperature and concentrated under reduced pressure. The corresponding compound of Formula 604 is isolated and purified.

Reaction Scheme 7



Preparation of Formula 604

[0075] As an alternative to Steps 3 and 4 of Reaction Scheme 6, acylation of primary amines of Formula 602, followed by acetic acid mediated cyclization, can proceed without isolation of the intermediate amides to provide the target compound of Formula 604. This route is shown in Reaction Scheme 7.

[0076] More specifically, to a solution of a compound of Formula 602 in a polar, aprotic solvent such as dichloromethane is added an excess, preferably about two equivalents of an amine base, such as triethylamine, followed by about an equivalent of an acid chloride. The resultant solution is stirred at ambient temperature for 2 hours, then evaporated under reduced pressure. The resultant solid is treated with glacial acetic acid, then the resultant suspension is heated at reflux for about 48 hours. The reaction is cooled to ambient temperature then evaporated under reduced pressure. The corresponding compound of Formula 604 is isolated and purified.

Preferred Processes and Last Steps

[0077] A compound of Formula I is contacted with a pharmaceutically acceptable acid to form the corresponding acid addition salt.

[0078] A pharmaceutically acceptable acid addition salt of Formula I is contacted with a base to form the corresponding free base of Formula I.

Preferred Compounds

[0079] When considering the compounds of Formula I, in a preferred embodiment R_1 is selected from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted heteroaryl, optionally substituted aralkyl, and optionally substituted heteroaralkyl.

[0080] In a more preferred embodiment R_1 is selected from hydrogen, optionally substituted lower alkyl, optionally substituted benzyl, optionally substituted naphthylmethyl, and optionally substituted phenyl.

[0081] In a most preferred embodiment R_1 is chosen from hydrogen, ethyl, propyl, methoxyethyl, naphthyl, phenyl, bromophenyl, chlorophenyl, methoxyphenyl, ethoxyphenyl, tolyl, dimethylphenyl, chorofluorophenyl, methylchlorophenyl, ethylphenyl, phenethyl, benzyl, chlorobenzyl, methylbenzyl, methoxybenzyl, cyanobenzyl, hydroxybenzyl, tetrahydrofuranylmethyl, dichlorobenzyl, furanylmethyl, dimethylbenzyl, dimethoxybenzyl, naphthylmethyl, (ethoxycarbonyl)ethyl, 3-benzo[1,3]dioxol-5-ylmethyl, benzyloxybenzyl, phenoxybenzyl, benzofuranylmethyl, and

3-3-methoxy-benzo[d]isoxazol-7-ylmethyl.

[0082] When considering the compounds of Formula I, in a preferred embodiment R_2 is hydrogen or optionally substituted alkyl. As will be appreciated by those skilled in the art, the compounds described herein possess a potentially chiral center at the carbon to which R_2 is attached. Thus, the R_2 position may comprise two substitution groups, R_2 and R_2'. The R_2 and R_2' groups may be the same or different; if different, the composition is chiral. When the R_2 and R_2' are different, preferred embodiments utilize only a single non-hydrogen R_2. The invention contemplates the use of pure enantiomers and mixtures of enantiomers, including racemic mixtures, although the use of the substantially optically pure eutomer will generally be preferred. In a preferred embodiment, the stereogenic center to which R_2 and R_2' are attached is of the R configuration.

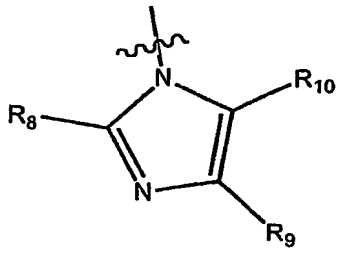
[0083] In a more preferred embodiment, R_2 is chosen from hydrogen, optionally substituted alkyl and optionally substituted aryl, and R_2' is hydrogen. In a most preferred embodiment R_2 is chosen from methyl, ethyl, propyl (particularly, c-propyl or i-propyl), butyl (particularly, t-butyl), methylthioethyl, methylthiomethyl, aminobutyl, (CBZ)aminobutyl, cyclohexylmethyl, benzyloxymethyl, methylsulfinylethyl, methylsulfinylmethyl, hydroxymethyl, phenyl, benzyl and indolylmethyl. Especially preferred is the R enantiomer where R_2 is i-propyl.

[0084] In other preferred embodiments R_4 is independently chosen from hydrogen, hydroxyl, halo (particularly chloro and fluoro), lower alkyl (particularly methyl), lower alkoxy (particularly methoxy) and cyano. More preferably, R_4 is hydrogen, halo, optionally substituted alkyl (particularly, methyl), optionally substituted aryl (particularly, phenyl), alkoxy (particularly, methoxy), cyano, substituted amino, carbamyl, aryloxy (particularly, phenoxy), heteroaryloxy (particularly, pyridinyloxy), heteroaryl (particularly, 2-oxo-2H-pyridinyl), optionally substituted N-heterocyclyl (particularly, morpholinyl or piperazinyl), or trifluoromethyl.

Compounds wherein R_3 is an Optionally Substituted Imidazolyl

[0085] Preferably, when R_3 is an optionally substituted imidazolyl, R_3 has the

formula:



wherein

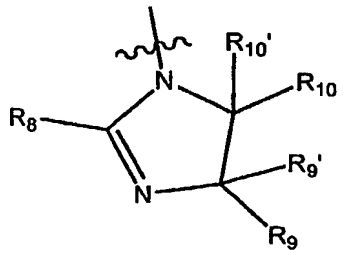
R_8 is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, and optionally substituted heteroaryl; and

R_9 and R_{10} are independently hydrogen, optionally substituted alkyl, optionally substituted aryl, or optionally substituted aralkyl.

[0086] Preferably, R_8 is aryl (preferably phenyl), substituted aryl (preferably lower alkyl-, lower alkoxy-, and/or halo-substituted phenyl), aralkyl (preferably benzyl and phenylvinyl), heteroaryl, substituted heteroaryl, heteroaralkyl, aralkoxy (preferably phenoxy lower alkyl), heteroaralkoxy, substituted aralkyl (preferably substituted benzyl and substituted styrenyl), substituted heteroaralkyl, substituted aralkoxy (preferably substituted phenoxy lower alkyl), or substituted heteroaralkoxy.

Compounds wherein R_3 is an Optionally Substituted Imidazolinyl

[0087] Preferably, when R_3 is an imidazoline, R_3 has the formula



wherein,

R_8 is chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, and optionally substituted heteroaryl; and

[0088] R_9, $R_{9'}$, R_{10}, and $R_{10'}$ are independently chosen from hydrogen, optionally substituted alkyl, optionally substituted aryl, and optionally substituted aralkyl.

[0089] Preferably, R_8 is aryl (preferably phenyl), substituted aryl (preferably lower alkyl-, lower alkoxy-, and/or halo-substituted phenyl), aralkyl (preferably benzyl and phenylvinyl), heteroaryl, substituted heteroaryl, heteroaralkyl, aralkoxy (preferably phenoxy lower alkyl), heteroaralkoxy, substituted aralkyl (preferably substituted benzyl and substituted styrenyl), substituted heteroaralkyl, substituted aralkoxy (preferably substituted phenoxy lower alkyl), or substituted heteroaralkoxy.

[0090] Preferably, R_9, $R_{9'}$, R_{10}, and $R_{10'}$ are independently selected from the group consisting of hydrogen and optionally substituted lower alkyl.

Compounds wherein R_3 is–NHR_5, –$NR_5(COR_6)$ –$NR_5(CH_2R_{6b})$

[0091] In a preferred embodiment, R_5 is chosen from optionally substituted alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaralkyl, and R_{11}-alkylene-, and R_{11} is chosen from alkoxy, amino, alkylamino, dialkylamino, carboxy, hydroxyl, and N-heterocyclyl.

[0092] In a more preferred embodiment, R_5 is selected from optionally substituted lower alkyl, optionally substituted cyclohexyl; phenyl substituted with hydroxy, lower alkoxy or lower alkyl; benzyl; heteroarylmethyl; heteroarylethyl; heteroarylpropyl and R_{11}-alkylene-, wherein R_{11} is amino, carboxy, lower alkylamino, di(lower alkyl)amino, lower alkoxy, hydroxyl, or N-heterocyclyl.

[0093] In a most preferred embodiment, R_5 is chosen from methyl, ethyl, propyl, butyl, cyclohexyl, carboxyethyl, carboxymethyl, methoxyethyl, hydroxyethyl, hydroxypropyl, dimethylaminoethyl, dimethylaminopropyl, diethylaminoethyl, diethylaminopropyl, aminopropyl, methylaminopropyl, 2,2-dimethyl-3-(dimethylamino)propyl, 1-cyclohexyl-4-(diethylamino)butyl, aminoethyl, aminobutyl, aminopentyl, aminohexyl, aminoethoxyethyl, isopropylaminopropyl,

diisopropylaminoethyl, 1-methyl-4-(diethylamino)butyl, (t-Boc)aminopropyl, hydroxyphenyl, benzyl, methoxyphenyl, methylmethoxyphenyl, dimethylphenyl, tolyl, ethylphenyl, (oxopyrrolidinyl)propyl, (methoxycarbonyl)ethyl, benzylpiperidinyl, pyridinylethyl, pyridinylmethyl, morpholinylethyl morpholinylpropyl, piperidinyl, azetidinylmethyl, azetidinylethyl, azetidinylpropyl pyrrolidinylethyl, pyrrolidinylpropyl, piperidinylmethyl, piperidinylethyl, imidazolylpropyl, imidazolylethyl, (ethylpyrrolidinyl)methyl, (methylpyrrolidinyl)ethyl, (methylpiperidinyl)propyl, (methylpiperazinyl)propyl, furanylmethyl and indolylethyl.

[0094] When considering the compounds of Formula I, in a preferred embodiment R_6 is selected from optionally substituted alkyl, optionally substituted aralkyl, optionally substituted heteroaralkyl, optionally substituted heteroaryl, optionally substituted aryl, R_7O- and R_7-NH-, and R_7 is chosen from optionally substituted alkyl and optionally substituted aryl.

[0095] In a more preferred embodiment, when R_6 is not R_7NH, R_6 is chosen from optionally substituted alkyl; aryl, including phenyl, biphenyl, and naphthyl; substituted aryl, including phenyl substituted with one or more halo, lower alkyl, lower alkoxy, nitro, carboxy, methylenedioxy or trifluoromethyl; benzyl; phenoxymethyl; halophenoxymethyl; phenylvinyl; heteroaryl; heteroaryl substituted with lower alkyl; and benzyloxymethyl.

[0096] In a most preferred embodiment, when R_6 is not R_7NH, R_6 is chosen from ethyl, propyl, chloropropyl, butoxy, heptyl, butyl, octyl, tridecanyl, (ethoxycarbonyl)ethyl, dimethylaminoethyl, dimethylaminomethyl, phenyl, naphthyl, halophenyl, dihalophenyl, cyanophenyl, halo(trifluoromethyl)phenyl, chlorophenoxymethyl, methoxyphenyl, carboxyphenyl, ethylphenyl, tolyl, biphenylyl, methylenedioxyphenyl, methylsulfonylphenyl, methoxychlorophenyl, chloronaphthyl, acetylphenyl, methylhalophenyl, trifluoromethylphenyl, butylphenyl, pentylphenyl, methylnitrophenyl, phenoxymethyl, dimethoxyphenyl, phenylvinyl, nitrochlorophenyl, nitrophenyl, dinitrophenyl, bis(trifluoromethyl)phenyl, benzyloxymethyl, benzyl, furanyl, benzofuranyl, pyridinyl, indolyl, methylpyridinyl, quinolinyl, picolinyl, pyrazolyl, morpholinomethyl, methylthiomethyl, methoxymethyl, and imidazolyl.

[0097] In a more preferred embodiment, when R_6 is R_7NH, R_7 is chosen from lower alkyl; cyclohexyl; phenyl; and phenyl substituted with halo, lower alkyl, loweralkoxy, or lower alkylthio.

[0098] In a most preferred embodiment, when R_6 is R_7NH, R_7 is isopropyl, butyl, cyclohexyl, phenyl, bromophenyl, dichlorophenyl, methoxyphenyl, ethylphenyl, tolyl, trifluoromethylphenyl or methylthiophenyl.

[0099] Preferably, R_{6b} is chosen from C_1-C_{13} alkyl; substituted lower alkyl; phenyl; naphthyl; phenyl substituted with halo, lower alkyl, lower alkoxy, nitro, methylenedioxy, or trifluoromethyl; biphenylyl, benzyl and heterocyclyl; and R_4 is chosen from lower alkyl; cyclohexyl; phenyl substituted with hydroxy, lower alkoxy or lower alkyl; benzyl; substituted benzyl; heterocyclyl; heteroarylmethyl; heteroarylethyl; heteroarylpropyl and R_{11}-alkylene, wherein R_{11} is hydroxy, di(lower alkyl)amino, (lower alkyl)amino, amino, lower alkoxy, or N-heterocyclyl. More preferably, R_{6b} is chosen from substituted phenyl, heterocyclyl and naphthyl and R_5 is chosen from subtituted benzyl, heterocyclyl and R_{11}-alkylene-. Most preferably, R_{6b} is chosen from halophenyl, polyhalophenyl, tolyl, dimethylphenyl, methoxyphenyl, dimethoxyphenyl, cyanophenyl, trifluoromethylphenyl, trifluoromethoxyphenyl, bis(trifluoromethyl)phenyl, carboxyphenyl, t-butylphenyl, methoxycarbonylphenyl, piperidinyl and naphthyl.

Compounds wherein R_3 is $-NR_5(SO_2R_{6a})$

[00100] Preferably, when R_3 is $-NR_5(SO_2R_{6a})$, R_5 is as described above and R_{6a} is chosen from C_1-C_{13} alkyl; phenyl; naphthyl; phenyl substituted with halo, lower alkyl, lower alkoxy, nitro, methylenedioxy, or trifluoromethyl; biphenylyl and heteroaryl. More preferably, R_{6a} is chosen from substituted phenyl and naphthyl.

Preferred Subgenus

[00101] In a particularly preferred subgenus of compounds of Formula I, R_1 is benzyl, halobenzyl, methoxylbenzyl, cyanobenzyl, or naphthylmethyl; R_2 is chosen from ethyl and propyl; R_2' is hydrogen; R_4 is methyl or phenyl; and R_3 is $-NHR_{6b}$ wherein R_{6b} is hydrogen.

[00102] In another particularly preferred subgenus of compounds of Formula I, R_1 is benzyl, halobenzyl, methoxylbenzyl, cyanobenzyl, or naphthylmethyl; R_2 is chosen from ethyl and propyl; R_2' is hydrogen; R_4 is methyl or phenyl; and R_3 is $-NR_5(COR_6)$ wherein R_5 is optionally substituted alkyl or R_{11}-alkylene wherein R_{11} is carboxy, hydroxy, di(lower alkylamino), (lower alkyl)amino, amino, pyrrolidino, piperidino, azetidino, imidazolyl and morpholino; and R_6 is optionally substituted aryl (preferably, p-tolyl).

[00103] In another particularly preferred subgenus of compounds of Formula I, R_1 is benzyl, halobenzyl, methoxylbenzyl, cyanobenzyl, or naphthylmethyl; R_2 is chosen from ethyl and propyl; R_2' is hydrogen; R_4 is methyl or phenyl; R_3 is $-NR_5(CH_2R_{6b})$ wherein R_5 is optionally substituted alkyl or R_{11}-alkylene wherein R_{11} is carboxy, hydroxy, di(lower alkylamino), (lower alkyl)amino, amino, pyrrolidino, piperidino, azetidino, imidazolyl or morpholino.; and R_{6b} is optionally substituted aryl.

[00104] In a particularly preferred subgenus of compounds of Formula I, R_1 is benzyl, halobenzyl, methoxylbenzyl, cyanobenzyl, or naphthylmethyl; R_2 is chosen from ethyl and propyl; R_2' is hydrogen; R_4 is methyl or phenyl; and R_3 is optionally substituted imidazolinyl wherein R_9, $R_{9'}$, R_{10} and $R_{10'}$ are independently hydrogen or optionally substituted alkyl (preferably optionally substituted lower alkyl); and R_8 is optionally substituted aryl (preferably, p-tolyl).

[00105] When R_3 is a sulfonamide, R_1 is most preferably chosen from lower alkyl, benzyl, substituted benzyl and substituted phenyl; R_2 is hydrogen or lower alkyl; R_2' is hydrogen; R_4 is methyl or phenyl; R_5 is R_{11}-alkylene; R_{6a} is substituted phenyl or naphthyl; and R_{11} is chosen from carboxy, hydroxy, di(lower alkylamino), (lower alkyl)amino, amino, azetidino, pyrrolidino, piperidino, imidazolyl and morpholino.

[00106] When R_3 is $-NHR_5$ or $-N(R_5)(CH_2R_{6b})$, R_1 is preferably chosen from hydrogen, optionally substituted lower alkyl, optionally substituted benzyl, optionally substituted phenyl, and optionally substituted naphthylmethyl; R_2 is chosen from hydrogen and optionally substituted lower alkyl and R_2' is hydrogen; R_4 is methyl or phenyl; R_{6b} is chosen from optionally substituted alkyl; optionally substituted phenyl; biphenylyl, optionally substituted aralkyl; and optionally substituted heterocyclyl; and R_5

is chosen from lower alkyl; cyclohexyl; optionally substituted phenyl; optionally substituted benzyl; heterocyclyl; heteroarylmethyl; heteroarylethyl; heteroarylpropyl and R_{11}-alkylene, wherein R_{11} is hydroxy, di(lower alkyl)amino, (lower alkyl)amino, amino, lower alkoxy, or N-heterocyclyl.

[00107] When R_3 is $-NHR_5$ or $-N(R_5)(CH_2R_{6b})$, R_1 is most preferably chosen from lower alkyl, optionally substituted benzyl, and optionally substituted phenyl; R_2 is hydrogen or optionally substituted lower alkyl; R_2' is hydrogen; R_{6b} is chosen from optionally substituted phenyl, optionally substituted heterocyclyl and naphthyl; R_5 is chosen from optionally substituted benzyl, optionally substituted heterocyclyl and R_{11}-alkylene-; R_4 is methyl or phenyl; and R_{11} is chosen from di(lower alkylamino), (lower alkyl)amino, amino, pyrrolidinyl, piperidinyl, azetidino, imidazolyl and morpholinyl. When R_{6b} is present, it is most preferably chosen from halophenyl, polyhalophenyl, tolyl, dimethylphenyl, methoxyphenyl, dimethoxyphenyl, cyanophenyl, trifluoromethylphenyl, trifluoromethoxyphenyl, bis(trifluoromethyl)phenyl, carboxyphenyl, t-butylphenyl, methoxycarbonylphenyl, piperidinyl and naphthyl.

[00108] When R_3 is an imidazole, in a particularly preferred subgenus of compounds of Formula I, R_1 is benzyl, halobenzyl, methoxylbenzyl, cyanobenzyl, or naphthylmethyl; R_2 is chosen from ethyl and propyl; $R_{2'}$ is hydrogen; R_4 is methyl or phenyl; and R_3 is preferably 2-(optionally substituted)-4,4-(optionally di-substituted)-4,5-dihydro-imidazol-1-yl; and more particularly, R_3 is 2-(optionally substituted aryl)-4,5-dihydro-imidazol-1-yl or 2-(optionally substituted aryl)-4,4-dimethyl-4,5-dihydro-imidazol-1-yl. In a preferred embodiment, R_3 is 2-(substituted phenyl)-4,5-dihydro-imidazol-1-yl or 2-(substituted phenyl)-4,4-dimethyl-4,5-dihydro-imidazol-1-yl.

[00109] When R_3 is an imidazole, in a particularly preferred subgenus of compounds of Formula I, R_1 is benzyl, halobenzyl, methoxylbenzyl, cyanobenzyl, or naphthylmethyl; R_2 is chosen from ethyl and propyl; $R_{2'}$ is hydrogen; R_4 is methyl or phenyl; and R_3 is preferably 2- and/or 4-substituted-imidazol-1-yl; more preferably, 2-substituted-imidazol-1-yl, such as 2-(optionally substituted phenyl)-imidazol-1-yl; and yet more preferably R_3 is 2-phenyl-imidazol-1-yl, 2-*p*-tolyl-imidazol-1-yl, 2-(4-fluoro-phenyl)-imidazol-1-yl, 2-(4-chloro-phenyl)-imidazol-1-yl, or 2-(3-fluoro-4-methyl-

phenyl)-imidazol-1-yl.

Utility, Testing and Administration

[00110] The compositions of the invention find use in a variety of applications. As will be appreciated by those in the art, mitosis may be altered in a variety of ways; that is, one can affect mitosis either by increasing or decreasing the activity of a component in the mitotic pathway. Stated differently, mitosis may be affected (e.g., disrupted) by disturbing equilibrium, either by inhibiting or activating certain components. Similar approaches may be used to alter meiosis.

[00111] In a preferred embodiment, the compositions of the invention are used to modulate mitotic spindle formation, thus causing prolonged cell cycle arrest in mitosis. By "modulate" herein is meant altering mitotic spindle formation, including increasing and decreasing spindle formation. By "mitotic spindle formation" herein is meant organization of microtubules into bipolar structures by mitotic kinesins. By "mitotic spindle dysfunction" herein is meant mitotic arrest and monopolar spindle formation.

[00112] The compositions of the invention are useful to bind to and/or modulate the activity of a mitotic kinesin, KSP. In a preferred embodiment, the KSP is human KSP, although KSP kinesins from other organisms may also be used. In this context, modulate means either increasing or decreasing spindle pole separation, causing malformation, i.e., splaying, of mitotic spindle poles, or otherwise causing morphological perturbation of the mitotic spindle. Also included within the definition of KSP for these purposes are variants and/or fragments of KSP. See U.S. Patent Application "Methods of Screening for Modulators of Cell Proliferation and Methods of Diagnosing Cell Proliferation States", filed Oct. 27, 1999 (U.S. Serial Number 09/428,156), hereby incorporated by reference in its entirety. In addition, other mitotic kinesins may be used in the present invention. However, the compositions of the invention have been shown to have specificity for KSP.

[00113] For assay of activity, generally either KSP or a compound according to the invention is non-diffusably bound to an insoluble support having isolated sample receiving areas (e.g., a microtiter plate, an array, etc.). The insoluble support may be

made of any composition to which the compositions can be bound, is readily separated from soluble material, and is otherwise compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include microtiter plates, arrays, membranes and beads. These are typically made of glass, plastic (e.g., polystyrene), polysaccharides, nylon or nitrocellulose, Teflon™, etc. Microtiter plates and arrays are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. The particular manner of binding of the composition is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusable. Preferred methods of binding include the use of antibodies (which do not sterically block either the ligand binding site or activation sequence when the protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the protein or agent on the surface, etc. Following binding of the protein or agent, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety.

[00114] The antimitotic agents of the invention may be used on their own to modulate the activity of a mitotic kinesin, particularly KSP. In this embodiment, the mitotic agents of the invention are combined with KSP and the activity of KSP is assayed. Kinesin activity is known in the art and includes one or more kinesin activities. Kinesin activities include the ability to affect ATP hydrolysis; microtubule binding; gliding and polymerization/depolymerization (effects on microtubule dynamics); binding to other proteins of the spindle; binding to proteins involved in cell-cycle control; serving as a substrate to other enzymes; such as kinases or proteases; and specific kinesin cellular activities such as spindle pole separation.

[00115] Methods of performing motility assays are well known to those of skill in the art. (See e.g., Hall, et al. (1996), Biophys. J., 71: 3467-3476, Turner et al., 1996, AnaL Biochem. 242 (1):20-5; Gittes et al., 1996, Biophys. J. 70(l): 418-29; Shirakawa et al., 1995, J. Exp. BioL 198: 1809-15; Winkelmann et al., 1995, Biophys. J. 68: 2444-53;

Winkelmann et al., 1995, Biophys. J. 68: 72S.)

[00116] Methods known in the art for determining ATPase hydrolysis activity also can be used. Preferably, solution based assays are utilized. U.S. application 09/314,464, filed May 18, 1999, hereby incorporated by reference in its entirety, describes such assays. Alternatively, conventional methods are used. For example, P_i release from kinesin can be quantified. In one preferred embodiment, the ATPase hydrolysis activity assay utilizes 0.3 M PCA (perchloric acid) and malachite green reagent (8.27 mM sodium molybdate II, 0.33 mM malachite green oxalate, and 0.8 mM Triton X-1 00). To perform the assay, 10 μL of reaction is quenched in 90 μL of cold 0.3 M PCA. Phosphate standards are used so data can be converted to mM inorganic phosphate released. When all reactions and standards have been quenched in PCA, 100 μL of malachite green reagent is added to the relevant wells in e.g., a microtiter plate. The mixture is developed for 10-15 minutes and the plate is read at an absorbance of 650 nm. If phosphate standards were used, absorbance readings can be converted to mM P_i and plotted over time. Additionally, ATPase assays known in the art include the luciferase assay.

[00117] ATPase activity of kinesin motor domains also can be used to monitor the effects of modulating agents. In one embodiment ATPase assays of kinesin are performed in the absence of microtubules. In another embodiment, the ATPase assays are performed in the presence of microtubules. Different types of modulating agents can be detected in the above assays. In a preferred embodiment, the effect of a modulating agent is independent of the concentration of microtubules and ATP. In another embodiment, the effect of the agents on kinesin ATPase can be decreased by increasing the concentrations of ATP, microtubules or both. In yet another embodiment, the effect of the modulating agent is increased by increasing concentrations of ATP, microtubules or both.

[00118] Agents that modulate the biochemical activity of KSP in vitro may then be screened in vivo. Methods for such agents in vivo include assays of cell cycle distribution, cell viability, or the presence, morphology, activity, distribution, or amount of mitotic spindles. Methods for monitoring cell cycle distribution of a cell population, for example, by flow cytometry, are well known to those skilled in the art, as are methods

for determining cell viability. See for example, U.S. Patent Application "Methods of Screening for Modulators of Cell Proliferation and Methods of Diagnosing Cell Proliferation States," filed Oct. 22, 1999, serial number 09/428,156, hereby incorporated by reference in its entirety.

[00119] In addition to the assays described above, microscopic methods for monitoring spindle formation and malformation are well known to those of skill in the art (see, e.g., Whitehead and Rattner (1998), J. Cell Sci. 111:2551-61; Galgio et al, (1996) J. Cell biol., 135:399-414).

[00120] The compositions of the invention inhibit the KSP kinesin. One measure of inhibition is IC_{50}, defined as the concentration of the composition at which the activity of KSP is decreased by fifty percent relative to a control. Preferred compositions have IC_{50}'s of less than about 1 mM, with preferred embodiments having IC_{50}'s of less than about 100 μM, with more preferred embodiments having IC_{50}'s of less than about 10 μM, with particularly preferred embodiments having IC_{50}'s of less than about 1 μM, and especially preferred embodiments having IC_{50}'s of less than about 100 nM, and with the most preferred embodiments having IC_{50}'s of less than about 10 nM. Measurement of IC_{50} is done using an ATPase assay.

[00121] Another measure of inhibition is K_i. For compounds with IC_{50}'s less than 1 μM, the K_i or K_d is defined as the dissociation rate constant for the interaction of the compounds described herein with KSP. Preferred compounds have K_i's of less than about 100 μM, with preferred embodiments having K_i's of less than about 10 μM, and particularly preferred embodiments having K_i's of less than about 1 μM and especially preferred embodiments having K_i's of less than about 100 nM, and with the most preferred embodiments having K_i's of less than about 10 nM. The K_i for a compound is determined from the IC_{50} based on three assumptions. First, only one compound molecule binds to the enzyme and there is no cooperativity. Second, the concentrations of active enzyme and the compound tested are known (i.e., there are no significant amounts of impurities or inactive forms in the preparations). Third, the enzymatic rate of the enzyme-inhibitor complex is zero. The rate (i.e., compound concentration) data are fitted to the equation:

$$V = V_{max}E_0 \left[I - \frac{(E_0 + I_0 + Kd) - \sqrt{(E_0 + I_0 + Kd)^2 - 4 E_0 I_0}}{2E_0} \right]$$

where V is the observed rate, V_{max} is the rate of the free enzyme, I_0 is the inhibitor concentration, E_0 is the enzyme concentration, and K_d is the dissociation constant of the enzyme-inhibitor complex.

[00122] Another measure of inhibition is GI_{50}, defined as the concentration of the compound that results in a decrease in the rate of cell growth by fifty percent. Preferred compounds have GI_{50}'s of less than about 1 mM. The level of preferability of embodiments is a function of their GI_{50} : those having GI_{50}'s of less than about 20 μM are more preferred; those having GI_{50}'s of 10 μM more so; those having GI_{50} of less than about 1 μM more so; those having GI_{50}'s of 100 nM more so; those having GI_{50} of less than about 10 nM even more so. Measurement of GI_{50} is done using a cell proliferation assay.

[00123] The compositions of the invention are used to treat cellular proliferation diseases. Disease states which can be treated by the methods and compositions provided herein include, but are not limited to, cancer (further discussed below), autoimmune disease, fungal disorders, arthritis, graft rejection, inflammatory bowel disease, proliferation induced after medical procedures, including, but not limited to, surgery, angioplasty, and the like. It is appreciated that in some cases the cells may not be in a hyper or hypo proliferation state (abnormal state) and still require treatment. For example, during wound healing, the cells may be proliferating "normally", but proliferation enhancement may be desired. Similarly, as discussed above, in the agriculture arena, cells may be in a "normal" state, but proliferation modulation may be desired to enhance a crop by directly enhancing growth of a crop, or by inhibiting the growth of a plant or organism which adversely affects the crop. Thus, in one embodiment, the invention herein includes application to cells or individuals afflicted or impending affliction with any one of these disorders or states.

[00124] The compositions and methods provided herein are particularly deemed

useful for the treatment of cancer including solid tumors such as skin, breast, brain, cervical carcinomas, testicular carcinomas, etc. More particularly, cancers that may be treated by the compositions and methods of the invention include, but are not limited to: Cardiac: sarcoma (angiosarcoma, fibrosarcoma, rhabdomyosarcoma, liposarcoma), myxoma, rhabdomyoma, fibroma, lipoma and teratoma; Lung: bronchogenic carcinoma (squamous cell, undifferentiated small cell, undifferentiated large cell, adenocarcinoma), alveolar (bronchiolar) carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesothelioma; Gastrointestinal: esophagus (squamous cell carcinoma, adenocarcinoma, leiomyosarcoma, lymphoma), stomach (carcinoma, lymphoma, leiomyosarcoma), pancreas (ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, carcinoid tumors, vipoma), small bowel (adenocarcinoma, lymphoma, carcinoid tumors, Karposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma, fibroma), large bowel (adenocarcinoma, tubular adenoma, villous adenoma, hamartoma, leiomyoma); Genitourinary tract: kidney (adenocarcinoma, Wilm's tumor (nephroblastoma), lymphoma, leukemia), bladder and urethra (squamous cell carcinoma, transitional cell carcinoma, adenocarcinoma), prostate (adenocarcinoma, sarcoma), testis (seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors, lipoma); Liver: hepatoma (hepatocellular carcinoma), cholangiocarcinoma, hepatoblastoma, angiosarcoma, hepatocellular adenoma, hemangioma; Bone: osteogenic sarcoma (osteosarcoma), fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma (reticulum cell sarcoma), multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma (osteocartilaginous exostoses), benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors; Nervous system: skull (osteoma, hemangioma, granuloma, xanthoma, osteitis deformans), meninges (meningioma, meningiosarcoma, gliomatosis), brain (astrocytoma, medulloblastoma, glioma, ependymoma, germinoma (pinealoma), glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma, congenital tumors), spinal cord neurofibroma, meningioma, glioma, sarcoma); Gynecological: uterus (endometrial

carcinoma), cervix (cervical carcinoma, pre-tumor cervical dysplasia), ovaries (ovarian carcinoma (serous cystadenocarcinoma, mucinous cystadenocarcinoma, unclassified carcinoma), granulosa-thecal cell tumors, Sertoli-Leydig cell tumors, dysgerminoma, malignant teratoma), vulva (squamous cell carcinoma, intraepithelial carcinoma, adenocarcinoma, fibrosarcoma, melanoma), vagina (clear cell carcinoma, squamous cell carcinoma, botryoid sarcoma (embryonal rhabdomyosarcoma), fallopian tubes (carcinoma); Hematologic: blood (myeloid leukemia (acute and chronic), acute lymphoblastic leukemia, chronic lymphocytic leukemia, myeloproliferative diseases, multiple myeloma, myelodysplastic syndrome), Hodgkin's disease, non-Hodgkin's lymphoma (malignant lymphoma); Skin: malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Karposi's sarcoma, moles dysplastic nevi, lipoma, angioma, dermatofibroma, keloids, psoriasis; and Adrenal glands: neuroblastoma. Thus, the term "cancerous cell" as provided herein, includes a cell afflicted by any one of the above identified conditions.

[00125] Accordingly, the compositions of the invention are administered to cells. By "administered" herein is meant administration of a therapeutically effective dose of the mitotic agents of the invention to a cell either in cell culture or in a patient. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for systemic versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art. By "cells" herein is meant almost any cell in which mitosis or meiosis can be altered.

[00126] A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and other organisms. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

[00127] Mitotic agents having the desired pharmacological activity may be

administered in a physiologically acceptable carrier to a patient, as described herein. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways as discussed below. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. The agents may be administered alone or in combination with other treatments, i.e., radiation, or other chemotherapeutic agents.

[00128] In a preferred embodiment, the pharmaceutical compositions are in a water soluble form, such as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.

[00129] The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic

pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

[00130] The administration of the mitotic agents of the present invention can be done in a variety of ways as discussed above, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the anti-mitotic agents may be directly applied as a solution or spray.

[00131] To employ the compounds of the invention in a method of screening for compounds that bind to KSP kinesin, the KSP is bound to a support, and a compound of the invention (which is a mitotic agent) is added to the assay. Alternatively, the compound of the invention is bound to the support and KSP is added. Classes of compounds among which novel binding agents may be sought include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for candidate agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

[00132] The determination of the binding of the mitotic agent to KSP may be done in a number of ways. In a preferred embodiment, the mitotic agent (the compound of the invention) is labeled, for example, with a fluorescent or radioactive moiety and binding determined directly. For example, this may be done by attaching all or a portion of KSP to a solid support, adding a labeled mitotic agent (for example a compound of the invention in which at least one atom has been replaced by a detectable isotope), washing off excess reagent, and determining whether the amount of the label is that present on the

solid support. Various blocking and washing steps may be utilized as is known in the art.

[00133] By "labeled" herein is meant that the compound is either directly or indirectly labeled with a label which provides a detectable signal, e.g., radioisotope, fluorescent tag, enzyme, antibodies, particles such as magnetic particles, chemiluminescent tag, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

[00134] In some embodiments, only one of the components is labeled. For example, the kinesin proteins may be labeled at tyrosine positions using ^{125}I, or with fluorophores. Alternatively, more than one component may be labeled with different labels; using ^{125}I for the proteins, for example, and a fluorophor for the mitotic agents.

[00135] The compounds of the invention may also be used as competitors to screen for additional drug candidates. "Candidate bioactive agent" or "drug candidate" or grammatical equivalents as used herein describe any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc., to be tested for bioactivity. They may be capable of directly or indirectly altering the cellular proliferation phenotype or the expression of a cellular proliferation sequence, including both nucleic acid sequences and protein sequences. In other cases, alteration of cellular proliferation protein binding and/or activity is screened. Screens of this sort may be performed either in the presence or absence of microtubules. In the case where protein binding or activity is screened, preferred embodiments exclude molecules already known to bind to that particular protein, for example, polymer structures such as microtubules, and energy sources such as ATP. Preferred embodiments of assays herein include candidate agents which do not bind the cellular proliferation protein in its endogenous native state termed herein as "exogenous" agents. In another preferred embodiment, exogenous agents further exclude antibodies to KSP.

[00136] Candidate agents can encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a

molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding and lipophilic binding, and typically include at least an amine, carbonyl, hydroxyl, ether, or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

[00137] Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

[00138] Competitive screening assays may be done by combining KSP and a drug candidate in a first sample. A second sample comprises a mitotic agent, KSP and a drug candidate. This may be performed in either the presence or absence of microtubules. The binding of the drug candidate is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of an agent capable of binding to KSP and potentially modulating its activity. That is, if the binding of the drug candidate is different in the second sample relative to the first sample, the drug candidate is capable of binding to KSP.

[00139] In a preferred embodiment, the binding of the candidate agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to KSP, such as an antibody, peptide,

binding partner, ligand, etc. Under certain circumstances, there may be competitive binding as between the candidate agent and the binding moiety, with the binding moiety displacing the candidate agent.

[00140] In one embodiment, the candidate agent is labeled. Either the candidate agent, or the competitor, or both, is added first to KSP for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C.

[00141] Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high throughput screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

[00142] In a preferred embodiment, the competitor is added first, followed by the candidate agent. Displacement of the competitor is an indication the candidate agent is binding to KSP and thus is capable of binding to, and potentially modulating, the activity of KSP. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the candidate agent is labeled, the presence of the label on the support indicates displacement.

[00143] In an alternative embodiment, the candidate agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate the candidate agent is bound to KSP with a higher affinity. Thus, if the candidate agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate the candidate agent is capable of binding to KSP.

[00144] It may be of value to identify the binding site of KSP. This can be done in a variety of ways. In one embodiment, once KSP has been identified as binding to the mitotic agent, KSP is fragmented or modified and the assays repeated to identify the necessary components for binding.

[00145] Modulation is tested by screening for candidate agents capable of

modulating the activity of KSP comprising the steps of combining a candidate agent with KSP, as above, and determining an alteration in the biological activity of KSP. Thus, in this embodiment, the candidate agent should both bind to KSP (although this may not be necessary), and alter its biological or biochemical activity as defined herein. The methods include both in vitro screening methods and in vivo screening of cells for alterations in cell cycle distribution, cell viability, or for the presence, morpohology, activity, distribution, or amount of mitotic spindles, as are generally outlined above.

[00146] Alternatively, differential screening may be used to identify drug candidates that bind to the native KSP, but cannot bind to modified KSP.

[00147] Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

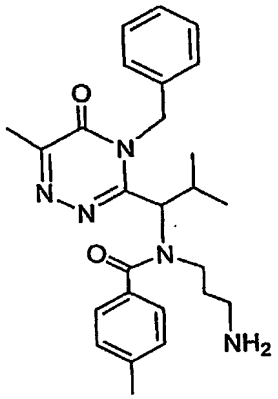
[00148] A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

[00149] The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

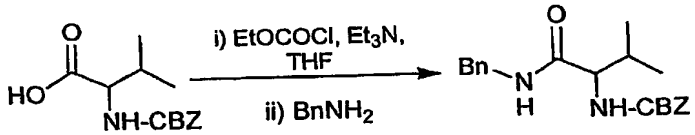
EXAMPLES

[00150] The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

Example 1



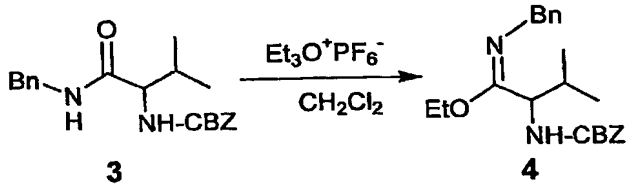
Experimental Section:



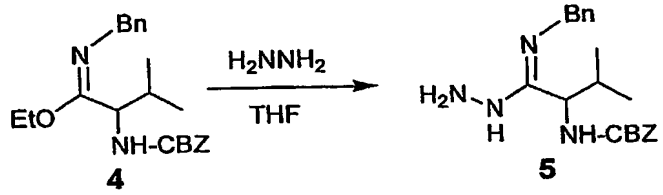
2 **3**

To a solution of CBZ-Valine (**2**, 50 g, 200 mmol) in THF (700 mL) were added ethyl chloroformate (23 mL, 240 mmol) and triethylamine (33.5 mL, 240 mmol) at 0 °C. The reaction mixture was stirred under nitrogen. After 1 hour, benzylamine (26.2 mL, 240 mmol) was added over 5 minutes. Upon completion of addition, the reaction solution was allowed to warm to room temperature. After 1 hour, the reaction mixture was filtered. The precipitate was washed with water and THF, and dried *in vacuo* to give **3** (60 g,

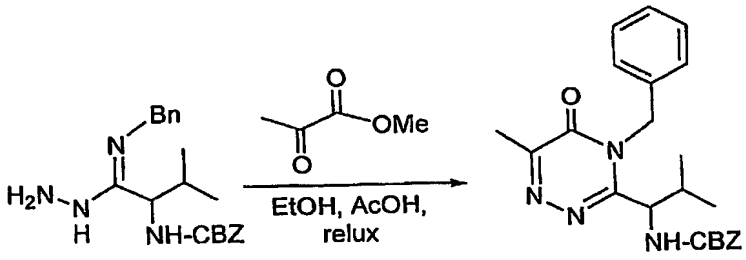
88%) as a white solid. LRMS ($M+H^+$) *m/z* 341.1.



To a suspension of **3** (20 g, 59 mmol) in dichloromethane (500 mL) was added triethyloxonium hexafluorophosphate (25 g, 100 mmol).The resulting mixture was stirred for 14 hours. The reaction mixture was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to give **4** (19 g), which was used in the next step without further purification. LRMS ($M+H^+$) *m/z* 369.1.

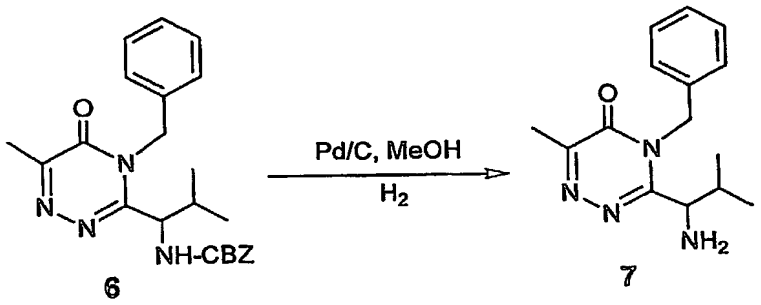


To a solution of above crude **4** (4 g, ~10.85 mmol) in THF (30 mL) was added hydrazine (1.0 M in THF, 20 mL). The resulting mixture was stirred for 4 hours. The solution was concentrated and dried *in vacuo* to give **5** (3.8 g) as a pale-yellow solid, which was used in the next step without further purification. LRMS ($M+H^+$) *m/z* 355.1.



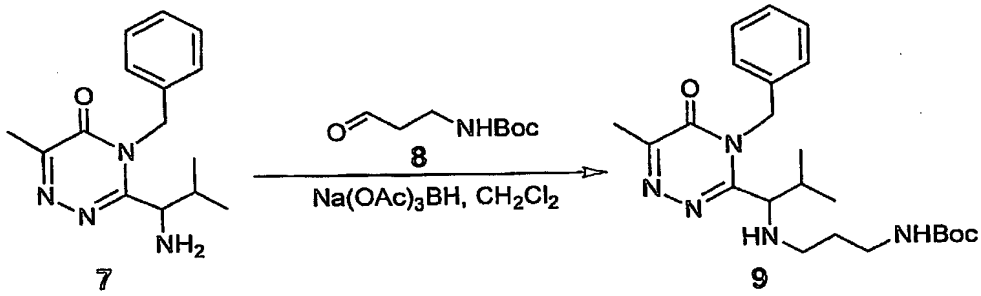
A solution of above crude **5** (3.5 g, ~9.85 mmol) and methyl pyruvate (1.4 mL, 15 mmol) in EtOH was refluxed for 4 hours. AcOH (800 μL) was added. Refluxing continued for

additional 6 hours. The reaction mixture was concentrated. The resulting residue was purified via flash column chromatography using a mixture of ethyl acetate and hexane as eluent to give 6 (1.0 g, 24% from 3) as a white solid. LRMS ($M+H^+$) *m/z* 407.1.



6 7

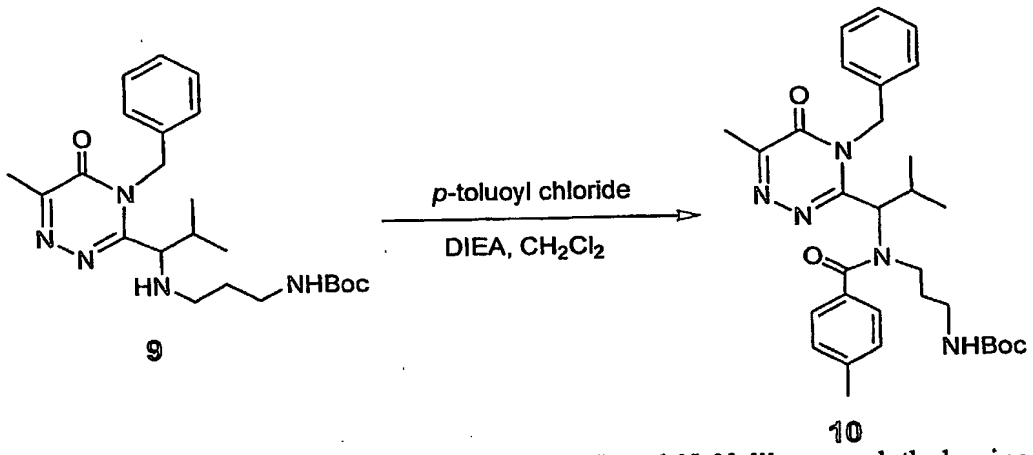
A solution of 6 (500 mg, 1.23 mmol) in MeOH (20 mL) was stirred under a stream of H_2 (30 psi) in the presence of 10% Pd/C (50 mg) for 40 minutes. The catalyst was removed by filtration through a PTFE (0.45 um) filter and the solvent evaporated to give 7 (400 mg), which was used in the next step without further purification. LRMS ($M+H^+$) *m/z* 273.1.



7 9

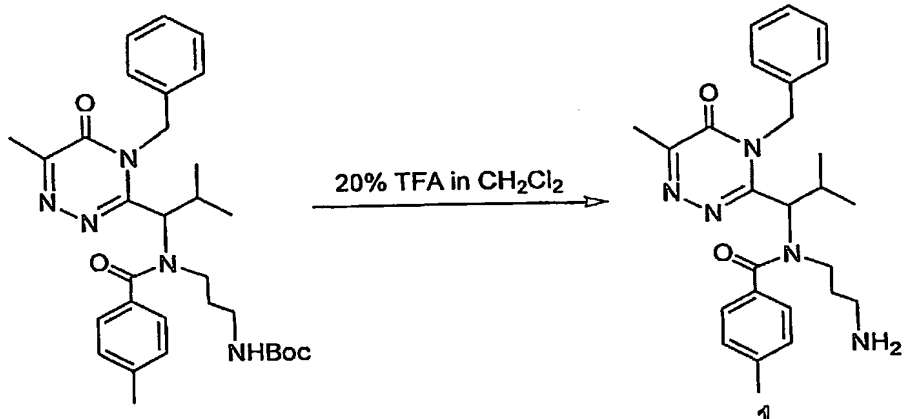
To a solution of above crude 7 (400 mg, ~1.47 mmol) in dichloromethane (35 mL) at 0 °C was added aldehyde 8 (330 mg, 1.91 mmol) and sodium triacetoxyborohydride (406 mg, 1.91 mmol), successively. The resulting mixture was stirred under nitrogen for 14 hours, followed by addition of saturated $NaHCO_3$ (20 mL). The organic layer was separated, and the aqueous phase was extracted with dichloromethane (2 x 50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated to give 9 (700 mg) as a yellow oil, which was used in the next step without further purification. LRMS ($M+H^+$) *m/z*

430.2.



9 10

To a solution of above crude 9 (350 mg, 0.82 mmol) and *N*, *N*-diisopropylethylamine (242 μL, 1.39 mmol) in dichloromethane (12 mL) at 0 °C was added *p*-toluoyl chloride (183 μL, 1.39 mmol). The resulting solution was stirred under nitrogen at room temperature for 20 hours. The solution was concentrated. The resulting residue was purified on RP-HPLC using a mixture of acetonitrile and H_2O to give 9 (80 mg, 36% from 6). LRMS ($M+H^+$) *m/z* 548.2.



10 1

To a solution of 10 (40 mg, 0.073 mmol) in dichloromethane (4 mL) was added trifluoroacetic acid (1 mL). The resulting solution was stirred at room temperature for 1 hour and then concentrated under reduced pressure. The residue was dried under high vacuum

and dissolved in dichloromethane (15 mL). It was washed with saturated $NaHCO_3$ (15 mL), and the aqueous phase was extracted with dichloromethane (2 x 25 mL). The combined organic layers were dried over Na_2SO_4, and concentrated to give **1** (20 mg, 62%) as a white solid, which was fully characterized with ^{1}H-NMR and LC/MS analysis (LRMS ($M+H^+$) *m/z* 448.2)

Example 2

Inhibition of Cellular Viability in Tumor Cell Lines Treated with KSP Inhibitors.

[00151] Materials and Solutions:

- Cells: SKOV3, Ovarian Cancer (human).
- Media: Phenol Red Free RPMI + 5% Fetal Bovine Serum + 2mM L-glutamine.
- Colorimetric Agent for Determining Cell Viability: Promega MTS tetrazolium compound.
- Control Compound for max cell kill: Topotecan, 1μM.

Procedure: Day 1 - Cell Plating:

[00152] Adherent SKOV3 cells are washed with 10mLs of PBS followed by the addition of 2mLs of 0.25% trypsin and incubation for 5 minutes at 37°C. The cells are rinsed from the flask using 8 mL of media (phenol red-free RPMI+ 5%FBS) and transferred to fresh flask. Cell concentration is determined using a Coulter counter and the appropriate volume of cells to achieve 1000 cells/100μL is calculated. 100 μL of media cell suspension (adjusted to 1000 cells/100 μL) is added to all wells of 96-well plates, followed by incubation for 18 to 24 hours at 37°C, 100% humidity, and 5% CO_2, allowing the cells to adhere to the plates.

Procedure: Day 2 – Compound Addition:

[00153] To one column of the wells of an autoclaved assay block are added an initial 2.5 μL of test compound(s) at 400X the highest desired concentration. 1.25 μL of

400X (400μM) Topotecan is added to other wells (ODs from these wells are used to subtract out for background absorbance of dead cells and vehicle). 500 μL of media without DMSO are added to the wells containing test compound, and 250 μL to the Topotecan wells. 250 μL of media + 0.5% DMSO is added to all remaining wells, into which the test compound(s) are serially diluted. By row, compound-containing media is replica plated (in duplicate) from the assay block to the corresponding cell plates. The cell plates are incubated for 72hours at 37°C, 100% humidity, and 5% CO_2.

Procedure: Day 4 – MTS Addition and OD Reading:

[00154] The plates are removed from the incubator and 40 μl MTS / PMS is added to each well. Plates are then incubated for 120 minutes at 37°C, 100% humidity, 5%CO_2, followed by reading the ODs at 490nm after a 5 second shaking cycle in a ninety-six well spectrophotometer.

Data Analysis

[00155] The normalized % of control (absorbance- background) is calculated and an XLfit is used to generate a dose-response curve from which the concentration of compound required to inhibit viability by 50% is determined. The compounds of the present invention show activity when tested by this method.

Example 3

Monopolar Spindle Formation following Application of a KSP Inhibitor

[00156] Human tumor cells Skov-3 (ovarian) were plated in 96-well plates at densities of 4,000 cells per well, allowed to adhere for 24 hours, and treated with various concentrations of the test compounds for 24 hours. Cells were fixed in 4% formaldehyde and stained with antitubulin antibodies (subsequently recognized using fluorescently-labeled secondary antibody) and Hoechst dye (which stains DNA).

[00157] Visual inspection revealed that the compounds caused cell cycle arrest in the prometaphase stage of mitosis. DNA was condensed and spindle formation had initiated, but arrested cells uniformly displayed monopolar spindles, indicating that there

was an inhibition of spindle pole body separation. Microinjection of anti-KSP antibodies also causes mitotic arrest with arrested cells displaying monopolar spindles.

Example 4

Inhibition of Cellular Proliferation in Tumor Cell Lines Treated with KSP Inhibitors.

[00158] Cells were plated in 96-well plates at densities from 1000-2500 cells/well of a 96-well plate and allowed to adhere/grow for 24 hours. They were then treated with various concentrations of drug for 48 hours. The time at which compounds are added is considered T_0. A tetrazolium-based assay using the reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (I.S> Patent No. 5,185,450) (see Promega product catalog #G3580, CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay) was used to determine the number of viable cells at T_0 and the number of cells remaining after 48 hours compound exposure. The number of cells remaining after 48 hours was compared to the number of viable cells at the time of drug addition, allowing for calculation of growth inhibition.

[00159] The growth over 48 hours of cells in control wells that had been treated with vehicle only (0.25% DMSO) is considered 100% growth and the growth of cells in wells with compounds is compared to this. KSP inhibitors inhibited cell proliferation in human ovarian tumor cell lines (SKOV-3).

[00160] A Gi_{50} was calculated by plotting the concentration of compound in μM vs the percentage of cell growth of cell growth in treated wells. The Gi_{50} calculated for the compounds is the estimated concentration at which growth is inhibited by 50% compared to control, i.e., the concentration at which:

$$100 \times [(Treated_{48} - T_0) / (Control_{48} - T_0)] = 50.$$

[00161] All concentrations of compounds are tested in duplicate and controls are averaged over 12 wells. A very similar 96-well plate layout and Gi_{50} calculation scheme is used by the National Cancer Institute (see Monks, et al., J. Natl. Cancer Inst. 83:757-766 (1991)). However, the method by which the National Cancer Institute quantitates cell number does not use MTS, but instead employs alternative methods.

Example 5

Calculation of IC_{50}:

[00162] Measurement of a composition's IC_{50} for KSP activity uses an ATPase assay. The following solutions are used: Solution 1 consists of 3 mM phosphoenolpyruvate potassium salt (Sigma P-7127), 2 mM ATP (Sigma A-3377), 1 mM IDTT (Sigma D-9779), 5 μM paclitaxel (Sigma T-7402), 10 ppm antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgC12 (VWR JT400301), and 1 mM EGTA (Sigma E3889). Solution 2 consists of 1 mM NADH (Sigma N8129), 0.2 mg/ml BSA (Sigma A7906), pyruvate kinase 7U/ml, L-lactate dehydrogenase 10 U/ml (Sigma P0294), 100 nM KSP motor domain, 50 μg/ml microtubules, 1 mM DTT (Sigma D9779), 5 μM paclitaxel (Sigma T-7402), 10 ppm antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgC12 (VWR JT4003-01), and 1 mM EGTA (Sigma E3889). Serial dilutions (8-12 two-fold dilutions) of the composition are made in a 96-well microtiter plate (Corning Costar 3695) using Solution 1. Following serial dilution each well has 50 μl of Solution 1. The reaction is started by adding 50 μl of solution 2 to each well. This may be done with a multichannel pipettor either manually or with automated liquid handling devices. The microtiter plate is then transferred to a microplate absorbance reader and multiple absorbance readings at 340 nm are taken for each well in a kinetic mode. The observed rate of change, which is proportional to the ATPase rate, is then plotted as a function of the compound concentration. For a standard IC_{50} determination the data acquired is fit by the following four parameter equation using a nonlinear fitting program (e.g., Grafit 4):

$$y = \frac{\text{Range}}{1 + \left(\frac{x}{IC_{50}}\right)^{s}} + \text{Background}$$

where y is the observed rate and x the compound concentration.

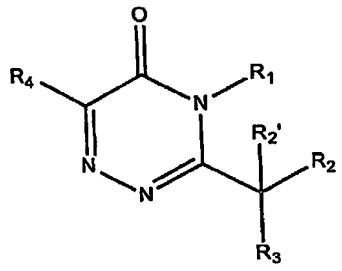
[00163] Other compounds of this class were found to inhibit cell proliferation, although GI_{50} values varied. GI_{50} values for the compounds tested ranged from 200 nM to greater than the highest concentration tested. By this we mean that although most of the

compounds that inhibited KSP activity biochemically did inhibit cell proliferation, for some, at the highest concentration tested (generally about 20 μM), cell growth was inhibited less than 50%. Many of the compounds have GI_{50} values less than 10 μM, and several have GI_{50} values less than 1 μM. Anti-proliferative compounds that have been successfully applied in the clinic to treatment of cancer (cancer chemotherapeutics) have GI_{50}'s that vary greatly. For example, in A549 cells, paclitaxel GI_{50} is 4 nM, doxorubicin is 63 nM, 5-fluorouracil is 1 μM, and hydroxyurea is 500 μM (data provided by National Cancer Institute, Developmental Therapeutic Program, http://dtp.nci.nih.gov/). Therefore, compounds that inhibit cellular proliferation at virtually any concentration may be useful. However, preferably, compounds will have GI_{50} values of less than 1 mM. More preferably, compounds will have GI_{50} values of less than 20 μM. Even more preferably, compounds will have GI_{50} values of less than 10 μM. Further reduction in GI_{50} values may also be desirable, including compounds with GI_{50} values of less than 1 μM. Some of the compounds of the invention inhibit cell proliferation with GI_{50} values from below 200 nM to below 10 nM.

All anhydrous solvents were purchased from Aldrich chemical company in SureSeal® containers. Most reagents were purchased from Aldrich Chemical Company. Reagents were adde and aqueous extractions performed with single or multichannel pipettors. Filtrations were perforr using Whatman/Polyfiltronics 24 well, 10 mL filtration blocks. Evaporation of volatile materials from the array was performed with a Labconco

ABSTRACT

1,2,4-Triazin-5-ones of Formula I are disclosed. They are useful for treating cellular proliferative diseases and disorders associated with KSP kinesin activity.



Formula I